

STRUCTURAL CHARACTERIZATION OF THE PRECURSOR OF MITOCHONDRIAL  
ASPARTATE AMINOTRANSFERASE AND ITS IN VITRO IMPORT INTO  
ISOLATED MITOCHONDRIA

INAUGURAL-DISSERTATION

zur

Erlangung der philosophischen Doktorwürde

vorgelegt der

Philosophischen Fakultät II

der

Universität Zürich

von

RENATA BEHRA

aus Arbon/TG

Begutachtet von Herrn Prof. Dr. Ph. Christen

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Dedicated to my mother



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- Biosynthesis of Aspartate Aminotransferases. Christen, P., Jaussi, R., Sonderegger, P., Gehring, H., Graf-Hausner, U., Behra, R., Flückiger, J., Skoda, R. (1984) in *Chemical and Biological Aspects of Vitamin B<sub>6</sub> Catalysis: Part B* (Evangelopoulos, A.E., ed ) pp. 17-25, Alan R. Liss, Inc., New York
- Inhibitors of Maturation and Degradation of the Precursor of Mitochondrial Aspartate Aminotransferase. Jaussi, R., Flückiger, J., Behra, R., and Christen, P. (1984) in *Chemical and Biological Aspects of Vitamin B<sub>6</sub> Catalysis: Part B* (Evangelopoulos, A.E., ed ) pp. 27-32, Alan R. Liss, Inc., New York
- Quaternary Structure of the Precursor of Mitochondrial Aspartate Aminotransferase. Behra, R., and Christen, P. (1984) 16th Annual Meeting of the Swiss Societies for Experimental Biology (USGEB), Zürich, 1984, *Experientia* 40, 617

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- In vitro Import into Mitochondria of the Precursor of Mitochondrial Aspartate Aminotransferase. Behra, R., and Christen, P.



## Abbreviations

|            |                                                     |
|------------|-----------------------------------------------------|
| AspAT      | Aspartate aminotransferase                          |
| mAspAT     | Mitochondrial AspAT                                 |
| cAspAT     | Cytosolic AspAT                                     |
| pre-mAspAT | Precursor of mAspAT                                 |
| CCCP       | Carbonyl cyanide m-chlorophenylhydrazone            |
| SDS        | Sodium dodecylsulfate                               |
| EDTA       | Ethylenediaminetetra-acetic acid                    |
| EGTA       | Ethyleneglycolbis(aminoethylether)tetra-acetic acid |

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## SUMMARY

This study was initiated in order to contribute to an understanding of the molecular mechanisms involved in the specific translocation of imported mitochondrial proteins from their site of synthesis in the cytosol into the mitochondria. Mitochondrial aspartate aminotransferase (mAspAT) like other imported mitochondrial proteins has been found previously to be synthesized as a precursor (pre-mAspAT) with higher molecular weight (Sonderegger, P., Jaussi, R., Christen, P. (1980) Biochem. Biophys. Res. Commun. 94, 1256-1260). In this thesis a structural characterization of pre-mAspAT and its import and processing by isolated mitochondria are reported.

Pre-mAspAT was synthesized in a reticulocyte lysate programmed with free polysomes from chicken liver in the presence of [ $^{35}\text{S}$ ]-methionine. Its sedimentation behavior was found to differ from that of the other in vitro synthesized proteins. On centrifugation of the translation mixture the major part of the precursor, in contrast to cytosolic AspAT and other proteins, cosedimented with the ribosomal fraction. The precursor was associated with polysomes containing the mRNA directing its synthesis rather than with endogenous ribosomes of the reticulocyte lysate.

The precursor in a reticulocyte lysate, similarly to the precursor in a homogenate of chicken embryo fibroblasts (Jaussi, R., Sonderegger, P., Flückiger, J., and Christen, P. (1982) J. Biol. Chem. 257, 13334-13340) and in contrast to the mature enzyme, was highly susceptible to trypsin indicating that the maturation of the precursor is accompanied by a conformational change.

Immunological studies were consistent with this notion. Competition experiments with an anti-denatured mAspAT antiserum indicated that pre-mAspAT contains epitopes which are present only in denatured mAspAT but not in nature mAspAT.

Both the precursor in the postribosomal supernatant of a reticulocyte lysate and the precursor in a postmitochondrial supernatant of chicken embryo fibroblasts were found to exist as low and high molecular weight complexes. The low molecular weight complexes probably are homo- or heterodimers of pre-mAspAT whereas the high molecular weight complexes with  $M_r > 300,000$  may represent heterooligomeric aggregates.

The import and processing of pre-mAspAT was reconstituted in vitro with isolated mitochondria from chicken heart. A reticulocyte lysate containing labeled translation products was incubated with intact mitochondria. In both cases about half of the precursor was converted to the mature form in a time-dependent process. Excess mature mAspAT did not compete with pre-mAspAT for its uptake into mitochondria. No conversion to the mature enzyme took place when pre-mAspAT was incubated with intact mitochondria in the presence of the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone or of the chelator o-phenanthroline which penetrates the mitochondrial inner membrane. In contrast, the chelator bathophenanthroline disulfonate which does not diffuse into the mitochondrial matrix did not inhibit the appearance of mature form. Thus, the translocation of pre-mAspAT follows the mechanism reserved for mitochondrial matrix and inner membrane proteins (for a review, see Hay, R., Böhni, P., and Gasser, S. (1984) *Biochem. Biophys. Acta* 779, 65-87) in that it occurs posttranslationally, requires an electrochemical gradient across the inner mitochondrial membrane for transport and a metal-dependent matrix protease for processing.

Like the import by isolated mitochondria processing by a mitochondrial extract converted also only about 50% of the total pre-mAspAT to the mature form. The addition of more mitochondria or more mitochondrial extract did not increase the fraction of imported or processed precursor. The coincident yields of import and processing together with the finding that the precursor in a homogenate from chicken embryo fibroblasts was neither imported nor processed by intact mitochondria or by a mitochondrial extract, respectively, indicate that under cell-free conditions the precursor might be subject to posttranslational modifications interfering with its import and processing.

## ZUSAMMENFASSUNG

Diese Studie ist unternommen worden mit dem Ziel, einen Beitrag zu leisten zum Verständnis der molekularen Mechanismen, die beteiligt sind an der spezifischen Translokation importierter mitochondrialer Proteine von ihrem Syntheseort im Zytosol in die Mitochondrien. Die mitochondriale Aspartat-Aminotransferase (mAspAT) wird wie andere importierte mitochondriale Proteine als Vorläuferprotein (Prä-mAspAT) mit höherem Molekulargewicht synthetisiert (Sonderegger, P., Jaussi, R., and Christen, P. (1980) Biochem. Biophys. Res. Commun. 94, 1256-1260). In der vorliegenden Schrift wird über eine strukturelle Charakterisierung der Prä-mAspAT und über ihren Import sowie ihr "Processing" durch isolierte Mitochondrien berichtet.

Die Prä-mAspAT wurde in einem mit freien Polysomen aus Hühnerleber programmierten Retikulozytenlysats in Gegenwart von [ $^{35}\text{S}$ ]-Methionin synthetisiert. Ihr Sedimentationsverhalten erwies sich als verschieden von dem der anderen in vitro synthetisierten Proteine. Nach Zentrifugation einer Translationsmischung cosedimentierte der Hauptteil des Vorläuferproteins im Gegensatz zur zytosolischen AspAT und zu anderen Proteinen mit der ribosomalen Fraktion. Das Vorläuferprotein war mit den Polysomen assoziiert, welche die Synthese leitende mRNA enthielten, und nicht mit den endogenen Ribosomen des Retikulozytenlysats.

Im Gegensatz zum reifen Enzym ist der Vorläufer in einem Retikulozytenlysats ähnlich wie der Vorläufer in einem Homogenat von Hühnerembryo-Fibroblasten (Jaussi, R., Sonderegger, P., Flückiger, J., and Christen, P. (1982) J. Biol. Chem. 257, 13334-13340) hochempfindlich gegenüber Trypsin. Offenbar ist die Reifung des Vorläufers von einer Konformationsänderung begleitet.

Immunologische Studien stimmen mit dieser Feststellung überein. Konkurrenzexperimente mit einem Antiserum gegen denaturierte mAspAT zeigten, dass Prä-mAspAT Epitope enthält, welche nur in der denaturierten nicht aber in der nativen mAspAT vorkommen.

Der Vorläufer in einem postribosomalen Ueberstand eines Retikulozytenlysats und auch der Vorläufer in einem postmitochondrialen Ueberstand von Hühnerembryo-Fibroblasten liegen teils als Komplexe mit niedrigem Molekulargewicht und teils als Komplexe mit hohem Molekulargewicht vor. Die Komplexe mit niedrigem Molekulargewicht sind wahrscheinlich Homo- oder Heterodimere der Prä-mAspAT, während die Komplexe mit hohem Molekulargewicht mit  $M_r > 300'000$  heterooligomeren Aggregaten entsprechen könnten.

Der Import und das "Processing" der Prä-mAspAT wurden in vitro mit isolierten Mitochondrien aus Hühnerherz rekonstituiert. Ein Retikulozytenlysatsatz oder ein postribosomaler Ueberstand eines Retikulozytenlysats, welche markierte Translationsprodukte enthielten, wurden mit intakten Mitochondrien inkubiert. In beiden Fällen, wurde ungefähr die Hälfte des Vorläufers in einem zeitabhängigen Prozess in die reife Form übergeführt. Ein Ueberschuss reife mAspAT kompetierte nicht mit der Prä-mAspAT für deren Aufnahme in die Mitochondrien. Es fand keine Umwandlung in die reife Form statt, wenn Prä-mAspAT in Gegenwart des Entkopplers Carbonylcyanid-m-chlorophenylhydrazon oder des Chelators o-Phenanthrolin, welcher die innere Mitochondrienmembran durchdringt, mit Mitochondrien inkubiert wurde. Im Gegensatz dazu hemmte der Chelator Bathophenanthrolindisulfonat, welcher nicht in die mitochondriale Matrix diffundiert, die Bildung der reifen mAspAT nicht. Demnach erfolgt die Translokation der Prä-mAspAT gemäss dem für Proteine der mitochondrialen Matrix und der inneren Membran geltenden Mechanismus (für einen Uebersichtsartikel, s. Hay, R., Böhni, P., and Gasser, S. (1984)

Biochem. Biophys. Acta 779, 65-87): Die Translokation geschieht posttranslationell und ist von einem elektrochemischen Gradienten an der inneren Mitochondrienmembran abhängig; für das "Processing" ist eine metallabhängige Protease der Matrix notwendig.

Wie der Import durch isolierte Mitochondrien wandelte auch das "Processing" durch einen mitochondrialen Extrakt nur etwa 50% der totalen Prä-mAspAT in die reife Form um. Die Zugabe von mehr Mitochondrien oder mehr mitochondrialem Extrakt führte nicht zu einer Zunahme des Anteils an importiertem oder "processiertem" Vorläufer. Die übereinstimmenden Import- und "Processing"-Ausbeuten zusammen mit dem erfolglosen Versuch, den in einem Homogenat von Hühnerembryo-Fibroblasten enthaltenen Vorläufer durch intakte Mitochondrien zu importieren oder durch einen mitochondrialen Extrakt zu "processieren", legen nahe, dass das Vorläuferprotein unter zellfreien Bedingungen posttranslationellen Modifikationen ausgesetzt ist, welche seinen Import und sein "Processing" beeinträchtigen.

## 1. INTRODUCTION

### 1.1 Biogenesis of Mitochondria

The study of mitochondria (for a review, see Ernster and Schatz, 1981) began in 1890 when Altman described the ubiquitous occurrence of "elementary organisms" in living cells which he called bioblasts and which were later renamed mitochondria (Benda, 1898). The following four decades were devoted to the morphological and constitutional, as well as functional characterization of the organelles.

Early in the 1950's Ephrussi (1950) initiated the study of mitochondrial biogenesis. He isolated respiration deficient "petite" mutants of yeast and showed that the respiration deficiency in crosses between these mutants and normal strains did not obey Mendelian inheritance. This finding together with demonstrations that isolated mitochondria can incorporate amino acids into proteins (McLean et al., 1958; Rendi, 1959), that mitochondrial protein synthesis can be inhibited by antibiotics, e.g., chloramphenicol, which do not inhibit microsomal protein synthesis (Mager, 1960), and that mitochondria contain their own DNA (Schatz et al., 1960; Nass et al., 1963; Luck et al., 1964; VanBruggen et al., 1966) indicated that mitochondrial biogenesis is at least in part independent from nuclear genetic control.

On the other hand, Sherman and coworkers (1966) clearly demonstrated that the primary structure of iso-1-cytochrome c, a mitochondrial protein, is coded for by a typical chromosomal gene thus indicating that the structural genes for part of the intramitochondrial proteins are located outside the organelle. The reports that the products of mitochondrial protein synthesis

make up for only 5% of the total mitochondrial protein (Sebald et al., 1968) and the identification of mitochondrial enzymes whose synthesis was sensitive to cycloheximide (Henson et al., 1968) suggested that many mitochondrial proteins are synthesized in the cytosol under the control of nuclear genes and are subsequently transported into the mitochondria (Beattie et al., 1966).

It is now established that mitochondria are formed by the interplay between the mitochondrial and the nucleo-cytoplasmic genetic system. Mitochondrial DNA codes for 3 out of 7 subunits of cytochrome c oxidase, 3 out of the about 10 subunits of the oligomycin-sensitive ATPase complex, and 1 out of the about 9 subunits of the cytochrome bc<sub>1</sub> complex (for a review, see Borst et al., 1983). Thus, mitochondria do not synthesize complete enzymes but only a few subunits of oligomeric structures. In addition, mitochondrial DNA codes for two rRNAs and about all tRNAs. Many open reading frames are still unassigned to known proteins (unidentified reading frames = URFs). It has been suggested that some gene products of URFs may play a role in the processing of mitochondrial RNA. Analysis of the mitochondrial genetic system has revealed some unusual features. For example, different variants of the mitochondrial genetic code are used in different species; in many species these variants differ from the "universal code" (Borst et al., 1983). The genes in animal mitochondrial DNA are highly packed. In contrast, several yeast mitochondrial genes are split by introns (Grivell et al., 1983). Another unusual feature of the mitochondrial genetic system is the need of only 22 different tRNAs for faithful reading of all codons as well as the occurrence of point mutations in the gene coding for the large rRNA which confer resistance to chloramphenicol (Borst et al., 1984).

Altogether about a hundred proteins constituting the mitochondrial genetic system must be specified by nuclear genes. An early explanation for the existence of a mitochondrial genetic system was that mitochondrial DNA is needed for coding those proteins which are too hydrophobic to reach their final location in the mitochondrial membranes from the outside of the organelle. Another hypothesis purports that the mitochondrial genetic system is an evolutionary dead end. Mitochondria are assumed to have arrived in the eucaryotic cells as bacterial endosymbionts which transferred most of their genes to the nucleus of the cells, this transfer having stopped before it was complete (Borst and Grivell, 1978).

For an understanding of mitochondrial biogenesis the question of how proteins made in the cytosol reach their functional site in the mitochondria (i.e., the outer membrane, the intermembrane space, the inner membrane, or the matrix) is of decisive importance. Both mitochondrial membranes constitute a barrier for macromolecules. The outer membrane is permeable to molecules of 10,000 daltons or less. Such molecules, including small proteins can reach the intermembrane space of the mitochondria but most of them cannot pass through the inner membrane. The inner membrane is impermeable even to small molecules, i.e., lipid-insoluble nonelectrolytes with a molecular weight above 100 to 150 daltons, and to ions (Ernster and Kuylenstierna, 1970). As a consequence, cytoplasmically made mitochondrial proteins cannot enter the mitochondrion through diffusion.

Two main experimental approaches have been used to elucidate some key steps of the import process: Pulse-chase studies with intact cells, and incubation of mitochondrial proteins synthesized in vitro with isolated mitochondria. So far the

following steps have been identified: 1) Synthesis of a precursor protein by free polysomes; 2) binding of the precursor to the mitochondrial surface; 3) translocation of the precursor into or across one or both mitochondrial membranes; 4) proteolytic processing of the precursor to the mature protein. Depending on the functional site of the protein, variations within these steps have been found. (for a review, see Hay et al., 1984).

In all organisms, except in yeast under specific metabolic conditions (Ades and Butow, 1980a,b; Suissa and Schatz, 1982), all precursor proteins studied so far have been found to be synthesized on free polysomes (Sonderregger et al., 1982; for a review, see Doonan et al., 1984). This finding indicates that the precursor proteins are transported posttranslationally into the mitochondria in contrast to the cotranslational movement of proteins which are exported from the cells and which are synthesized on membrane-bound polysomes (Palade, 1975).

A vast majority of the proteins located in the inner membrane and in the matrix space have been found to be synthesized as precursors with a molecular weight larger than the corresponding mature proteins. Only a few such proteins are synthesized with the same molecular weight as the corresponding mature protein (Côté et al., 1979; Zimmermann and Neupert, 1980; Nabi and Omura, 1980; Gasser et al., 1982b; Teintze et al., 1982; Hampsey et al., 1983; Hatalova and Kolarov, 1983). For some precursors it has been demonstrated that the increased molecular weight corresponds to an N-terminal extension (Lewin et al., 1980; Mihara and Blobel, 1980; Viebrock et al., 1982; Kaput et al., 1982; Horwich et al., 1984; Suissa et al., 1984; Sadler et al., 1984; Maarse et al., 1984; Jaussi et al., 1985). The sole common feature among the N-terminal extensions sequenced so far is the presence of basic residues and the absence of acidic residues.

The amino-terminal presequence is required for the transport of precursor proteins into the mitochondria. When the presequence of the precursor of subunit V of cytochrome c oxidase was removed by incubation with the processing matrix protease, the resulting mature protein was not imported into mitochondria (Gasser et al., 1982b). Very recently, Hurt and coworkers (1984a,b) showed that the N-terminal extension of the precursor of subunit IV of cytochrome c oxidase contains the information to direct polypeptides across both mitochondrial membranes. By gene fusion experiments, the N-terminal presequence of the precursor of subunit IV of cytochrome c oxidase was attached to dihydrofolate reductase, a cytosolic protein. The fusion protein was transported into the mitochondrial matrix on incubation with isolated mitochondria and cleaved to a polypeptide of a molecular weight corresponding to that of dihydrofolate reductase. Apparently, the N-terminal extension without the participation of other structural features of the precursor protein suffices to direct a protein into the mitochondria.

When precursors synthesized in a cell-free system are incubated with isolated mitochondria they first interact with the outer mitochondrial membrane and are then translocated into the mitochondria (Zimmermann and Neupert, 1981; Hennig et al., 1983; Zwizinski et al., 1983; Gasser and Schatz, 1983; Riezman et al., 1983; Zwizinski et al., 1984). The translocation is dependent on the electrochemical potential across the inner mitochondrial membrane (Gasser et al., 1982a,b; Schleyer et al., 1982; Kolansky et al., 1982). Concomitantly or after translocation, precursors with an N-terminal extension are cleaved to the corresponding mature proteins by a chelator-sensitive protease which is located in the matrix space (Böhni et al., 1980,1983).

Proteins of the intermembrane space seem to be imported by different pathways. For cytochrome b, and cytochrome  $c_1$  a two-step import mechanism has been proposed (Gasser et al., 1982a; Ohashi et al., 1982; Daum et al., 1982). Both proteins are made as larger precursors. After interacting with the outer mitochondrial membrane at least part of the molecule is translocated across the inner membrane (this step is dependent on the electrochemical potential across the inner mitochondrial membrane) and is cleaved by the chelator-sensitive protease to a form which is intermediary in molecular weight between the primary translation product and the mature form. The intermediate which is probably bound to the outer face of the inner membrane is then cleaved to the mature form by a second protease (not chelator-sensitive). In the case of cytochrome  $c_1$ , the second proteolytic step requires heme. On the other hand, cytochrome c which is also an intermembrane space protein is not synthesized as larger precursor. It is imported as apocytochrome c without requiring an energized inner membrane. In the mitochondria it is then converted to holocytochrome c (Zimmermann et al., 1981).

Proteins destined to the outer mitochondrial membrane are not synthesized as larger precursors. After binding to the surface of the mitochondrion such proteins become integrated into the membrane and undergo a conformational change conferring protease resistance (Gasser and Schatz, 1983).

Formation of functional units requires that after import into the mitochondria the processed precursors assemble to oligomeric structures. Some proteins must form homooligomers, other enzymes are heterooligomers and require assembling of imported and mitochondrially made constituents. Besides the findings that an imported protein has reached its correct final location and that

it shares some physical properties with the native enzyme (Zimmermann and Neupert, 1980; Lewin and Norman, 1983; Kalousek et al., 1984), proper assembling of imported subunits into oligomers in mitochondria has not yet been demonstrated.

Although some individual steps of the import process have been identified it is still unknown how they interconnect in the mechanism of import. Topics for future research may include studies on how a precursor reaches the mitochondrion, identification of the putative import receptors and of extra-mitochondrial factors or structures involved in the import process, identification of structural features of the precursor required for its import and processing, mechanism of assembling of the imported subunits, and regulation of the import process. The isolation of the genes for mitochondrial proteins and the identification of individual components involved in the import process (Hennig and Neupert, 1981; Böhni et al., 1983; Ohta and Schatz, 1984) which can be analyzed individually in a reconstituted process may be expected to answer some of these open questions.

## 1.2 Biosynthesis of the Isoenzymes of Aspartate Amino-transferase

Two isoenzymes of aspartate aminotransferase occur in animal cells (for a review, see Braunstein, 1973) and probably in all higher eucaryotic cells. One isoenzyme is found exclusively in the mitochondrial matrix (mAspAT), the other isoenzyme is located in the cytosol (cAspAT). Apparently, the two isoenzymes do not have any organ- or tissue specific properties (Wada and Morino, 1964; Shrawder and Martinez-Carrion, 1973; Behra et al., 1981). The conclusion that both isoenzymes are coded for by nuclear DNA was reached from hybridization studies of human white

blood cells from individuals carrying variant forms of AspAT. The variants were inherited according to Mendelian laws (Davidson et al., 1970). By interspecies hybridization of human and other eucaryotic cells, the genes both of the cytosolic and the mitochondrial isoenzyme could be assigned to particular chromosomes (Creagan et al., 1973; McAlpine et al., 1975; van Heyningen et al., 1975).

The different intracellular location raises the question whether the two isoenzymes have different physiological roles. Actually, the metabolic functions of AspAT are not too clearly depictable. Many lines of experimental evidence suggest that AspAT is important in gluconeogenesis in directing the nitrogen flow in form of aspartate into the urea cycle, and in linking the urea and the citric acid cycle (for a review, see Cooper and Meister, 1985). In addition, it is generally accepted that AspAT as part of the malate-aspartate shuttle plays a role in the transfer of reducing equivalents from the cytosol into the mitochondria (Cooper and Meister, 1985). In the cytosol, NADH serves to reduce oxalacetate to malate which is then transferred into the mitochondria where it is reoxidized by  $\text{NAD}^+$  to oxalacetate thus regenerating NADH. Mitochondrial AspAT converts oxalacetate to aspartate which leaves the mitochondrion. In the cytosol, cAspAT converts aspartate back to oxalacetate. So far it is unclear why higher eucaryotic cells require two isoenzymes of AspAT, especially in the light of the fact that only the cytosolic form is present in lower eucaryotes like yeast (Hollenberger et al., 1970).

The two isoenzymes are structurally very similar. AspAT is a dimeric protein consisting of two identical subunits each of  $M_r \sim 45,000$ . The amino acid sequences of the two isoenzymes from pig, chicken and other species have been determined (for

a review, see Christen et al., 1985). A comparison of the primary structures revealed an intraspecies identity of 46% between the cytosolic and mitochondrial isoenzymes in chicken and of 48% between the isoenzymes in pig (Graf-Hausner et al., 1982). Clearly, the two isoenzymes are homologous proteins. Accordingly, the tertiary structures of the two isoenzymes virtually coincide (Ford et al., 1980; Arnone et al., 1982; Jansonius et al., 1985; Arnone et al., 1985).

The occurrence of two homologous heterotopic proteins which are both synthesized on cytosolic ribosomes raises the question what structural features enable the mitochondrial isoenzyme to reach its proper final location. In order to study the biogenesis of mAspAT, the protein was synthesized in a heterologous cell-free system. Translation of chicken heart mRNA in a rabbit reticulocyte lysate indicated that mAspAT is synthesized as a precursor (pre-mAspAT) with a higher molecular weight than the mature protein ( $\Delta M_r \sim 2,000$ ; Sonderegger et al., 1980). The primary translation product of cAspAT has the same molecular weight as the mature enzyme. The mitochondrial AspATs from rat and pig were reported to be also synthesized as precursors with higher molecular weight (Sakakibara et al., 1980; Sannia et al., 1982). Like the majority of other mitochondrial precursor proteins (see Introduction, 1.1), pre-mAspAT has been found to be synthesized exclusively by free polysomes (Sonderegger et al., 1982). This finding suggests that in vivo the precursor is imported posttranslationally into the mitochondria.

Additional experimental evidence for posttranslational import of pre-mAspAT came from the finding that a small precursor pool exists in chicken embryo fibroblasts pulsed with [ $^{35}\text{S}$ ]methionine (Jaussi et al., 1982). The import of precursor proteins can be blocked by dissipation of the electrochemical gradient through

the inner mitochondrial membrane (Gasser et al., 1982a; Schleyer et al., 1982; Gasser et al., 1982b) with uncouplers such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP). In fibroblast cells treated with CCCP, the translocation of pre-mAspAT was inhibited; under these conditions the precursor was found to accumulate in the cytosol (Jaussi et al., 1982) in accordance with its synthesis by free polysomes. The increase in its pool is maximally 10-fold because the accumulation is limited by a rapid specific degradation (Jaussi et al., 1982; Flückiger et al., 1982). The half-life of degradation of pre-mAspAT in CCCP-treated fibroblast cells was found to be  $\sim 5$  min. This value compared to the half-life of import of pre-mAspAT in untreated cells (30-60s) explains the increase of the precursor pool size by a factor of only 10. The physiological meaning of the degradation of pre-mAspAT in CCCP-treated cells is still unknown. Although it cannot be excluded that the degradation is induced by the CCCP-treatment itself, it appears possible that the degradation plays a role in regulating the size of the precursor pool in the cytosol. Pre-mAspAT accumulated in the cytosol of CCCP-treated fibroblasts can be quantitatively chased into the mitochondria after dilution of CCCP. This finding provides additional experimental evidence for a posttranslational import of pre-mAspAT into the mitochondria.

AspAT is a pyridoxal-5'-phosphate dependent enzyme. However, the translocation of pre-mAspAT into the mitochondria, including its processing, apparently does not require the coenzyme (Sharma and Gehring, 1985). The uptake of the coenzyme proceeds independently (Lui et al., 1982).

No further details on the molecular events leading to the movement of pre-mAspAT into the mitochondria are known. The additional N-terminal segment (Jaussi et al., 1985) alone or in

combination with other structural features of the protein, must provide the information for directing the precursor into the mitochondria. A role of structural features other than the extra-peptide is suggested from the occurrence of amino acid residues which are invariant specifically in the mitochondrial isoenzyme. These so-called m-residues were found to cluster in certain surface regions of the protein (Graf-Hausner et al., 1983). Recently, cDNA clones (Jaussi et al., 1985) and genomic clones (Juretić et al., 1985) coding for chicken mAspAT have been isolated. Expression of the cDNA combined with site-directed mutagenesis is expected to provide a new approach to the molecular mechanism of import.

The purpose of this study was to adduce additional insight into the biosynthesis of imported mitochondrial proteins. Mitochondrial AspAT seems a favorable object of study because both its primary structure (Graf-Hausner et al., 1983) and its spatial structure are known (Ford et al., 1980). At the beginning of this study it was already established that mAspAT is synthesized as a higher molecular weight precursor and the available experimental evidence suggested that pre-mAspAT is transported post-translationally into the mitochondria (see above). It had been noted that pre-mAspAT from chicken embryo fibroblasts is susceptible to mild protease treatment. In contrast, the isolated mature enzyme had proven to be highly resistant to trypsin and other proteases (Jaussi et al., 1982; Sandmeier and Christen, 1980). The present study attempted to ascribe the differences between the precursor and the mature form to differences in conformation or in quaternary structure. A major goal was the reconstitution of the import of pre-mAspAT with isolated mitochondria as direct proof of a posttranslational uptake mechanism. In addition, an in vitro import assay offers the following experimental advantages: (1) It allows to study separately the

effects of inhibitors on the translocation process on the one hand and on the processing to the mature enzyme on the other.

(2) Any compounds which do not penetrate intact cells, e.g., the mature enzyme, can be assayed as putative inhibitors of the import.

## 2. EXPERIMENTAL PROCEDURE

### 2.1 Preparation of Polysomes

Estradiol treatment of roosters for the preparation of polysomes from liver and disruption of liver tissue was as described (Sonderegger et al., 1982) except for the composition of the media. Medium A contained 20 mM Tris chloride, 75 mM potassium chloride, 100 mM potassium acetate, 5 mM magnesium acetate (pH 7.6). Medium B contained in addition to medium A 4 mg ml<sup>-1</sup> yeast RNA, 4 mg ml<sup>-1</sup> heparin, 200 mM sucrose, and 5 mM dithiothreitol. The tissue suspension was homogenized with a Dounce homogenizer by 6 strokes with the loosely fitting pestle A and two strokes with the tightly fitting pestle B. Subsequently, the homogenate was centrifuged in a Sorvall SS-34 fixed angle rotor at 10,000 rpm (12,000 x g<sub>max</sub>) for 5 min at 0°C. The supernatant was again centrifuged in the same rotor at 12,000 rpm (17,400 x g<sub>max</sub>) for 10 min at 0°C. For isolation of free polysomes, the supernatant was overlaid onto a discontinuous gradient comprising 6 ml each of 1.38 M and 2 M sucrose containing 40 mM Tris chloride, 25 mM potassium chloride, 5 mM magnesium acetate, 4 mg ml<sup>-1</sup> yeast RNA, 5 mM dithiothreitol (pH 7.6). The 1.38 M sucrose solution contained in addition 4 mg ml<sup>-1</sup> heparin. After centrifugation in a Sorvall T 865 rotor at 52,000 rpm (200,000 x g<sub>av</sub>) for 20 h at 2°C, the polysomal pellets were resuspended in polysome buffer (50 mM Tris chloride, 25 mM potassium chloride, 5 mM magnesium acetate, 2 mM dithiothreitol, pH 7.6) and stored at -70°C. To assess polysome size and integrity, their sedimentation profiles were analyzed on 15 to 33.2% sucrose isokinetic gradients as described (Sonderegger et al., 1982), except that the sucrose solutions contained the same salt concentrations as the polysome buffer, and centrifugation was

in a Kontron TST 41 rotor at 40,000 rpm ( $204,000 \times g_{av}$ ) for 40 min at 4°C.

## 2.2 Cell-free Protein Synthesis

Read-out translation of free polysomes in a reticulocyte lysate in the presence of [ $^{35}$ S]methionine was performed as described (Sonderegger et al., 1982) except that incubation was at 29°C for 40 min. Protein synthesis was stopped by chilling or, when specified, by the addition of 0.2 mM cycloheximide. Postribosomal supernatants were obtained by centrifuging the chilled translation mixture in a Kontron TST 60 rotor at 45,000 rpm ( $190,000 \times g_{av}$ ) for 1 h at 2°C.

## 2.3 Preparation of Samples for Gel Permeation Chromatography

For analysis of pre-mAspAT synthesized in vivo five Petri dishes of cultured chicken embryo fibroblasts were pulsed with  $0.3 \text{ mCi ml}^{-1}$  [ $^{35}$ S]methionine for 15 min at 37°C in the presence of 20  $\mu\text{M}$  carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Jaussi et al., 1982). The cells were harvested in the cold, centrifuged for 7 min at 2,400 rpm, and taken up in 300  $\mu\text{l}$  homogenization buffer containing 80  $\mu\text{g ml}^{-1}$  each of chymostatin, antipain, leupeptin, 175  $\mu\text{g ml}^{-1}$  aprotinin, 5 mM o-phenanthroline, 2 mM EGTA, 2 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 150 mM potassium chloride, 1 mM dithiothreitol, and 10 mM Tris chloride (pH 7.1). Homogenization was performed by 20 strokes with a Teflon pestle in a Potter-Elvehjem homogenizer at 1750 rpm and 4°C. A post-mitochondrial supernatant was obtained by centrifugation in an Eppendorf centrifuge for 10 min at 12,000 rpm ( $8000 \times g_{max}$ ) and 4°C.

The sample for chromatography of pre-mAspAT synthesized in a cell-free system was obtained by diluting the postribosomal supernatant of a [ $^{35}\text{S}$ ]methionine labeled reticulocyte lysate containing  $120\ \mu\text{g ml}^{-1}$  each of chymostatin, antipain, leupeptin and  $260\ \mu\text{g ml}^{-1}$  aprotinin, 1 mM dithiothreitol with one volume of 150 mM potassium chloride, 1 mM dithiothreitol, 10 mM Tris chloride (pH 7.4).

#### 2.4 Preparation of Samples for Sucrose Gradient Centrifugation

The sample of in vivo synthesized pre-mAspAT was prepared in the same way as the sample for gel permeation chromatography except that the homogenization buffer was adjusted to pH 7.4 and contained no dithiothreitol. The sample of in vitro synthesized pre-mAspAT was the same as for gel permeation chromatography except that the postribosomal supernatant was diluted with one volume of 150 mM potassium chloride, 10 mM Tris chloride (pH 7.4).

For analysis of newly processed mAspAT one Petri dish of chicken embryo fibroblasts was pulsed with  $0.2\ \text{mCi ml}^{-1}$  [ $^{35}\text{S}$ ]methionine for 90 min. The cells were harvested in 300  $\mu\text{l}$  sonication buffer containing  $40\ \mu\text{g ml}^{-1}$  each of chymostatin, antipain, leupeptin,  $87.5\ \mu\text{g ml}^{-1}$  aprotinin, 150 mM potassium chloride, 10 mM Tris chloride (pH 7.4). After treatment for 10 min in an ultrasonic ice-water bath at 35 kHz the sample was cleared by centrifugation for 10 min at 12,000 rpm ( $8000 \times g_{\text{max}}$ ) in an Eppendorf centrifuge.

## 2.5 Gel Permeation Chromatography

Samples (200  $\mu$ l) were chromatographed on a TSK-G 3000 SW column from LKB (7.5 x 600 mm) and eluted with 150 mM potassium chloride, 1 mM dithiothreitol, 10 mM Tris chloride (pH 7.1). The flow rate was 1 ml min<sup>-1</sup>. Fractions of 0.5 ml were collected and analyzed by immunoprecipitation.

## 2.6 Sucrose Gradient Centrifugation

Samples (200  $\mu$ l) were layered on 5 to 35% (w/w) sucrose gradients (11 ml) and centrifuged for 20 h at 2°C using a Kontron TST 41 rotor. The sucrose solution contained 150 mM potassium chloride, 10 mM Tris chloride (pH 7.4). In the case of the in vivo synthesized precursor, the sucrose solution contained 40  $\mu$ g ml<sup>-1</sup> each of chymostatin, antipain, leupeptin, 87.5  $\mu$ g ml<sup>-1</sup> aprotinin, 5 mM o-phenanthroline, 2 mM EGTA, 2 mM EDTA, and 2 mM phenylmethanesulfonyl fluoride. In the absence of the protease inhibitors the half-life of the precursor in the cell homogenate at 4°C was only 5 h. Fractions were collected by puncturing the gradient tubes at the bottom. Sucrose concentrations were determined in a Bausch and Lomb refractometer. The fractions were analyzed by immunoprecipitation.

## 2.7 In vitro Import into Mitochondria and Processing of pre-mAspAT

The incubation mixture for in vitro import contained 60  $\mu$ l of the labeled reticulocyte lysate ( $\sim 7 \times 10^6$  cpm of [<sup>35</sup>S]methionine incorporated into protein) or 80  $\mu$ l of a labeled postribosomal supernatant which had been adjusted to 210 mM sucrose, 110 mM

potassium acetate, and 1.5 mM magnesium acetate. Mitochondria were isolated from chicken heart as described (Behra et al., 1982) except that EGTA was omitted from the isolation medium. The freshly prepared mitochondria (250  $\mu$ g mitochondrial protein in 25  $\mu$ l isolation medium) were added to the translation products and incubated at 29°C for 1 h. The mitochondria were then reisolated by centrifugation in a SS-34 rotor at 12,500 rpm (15,000  $\times$   $g_{max}$ ) for 15 min at 4°C. To both the mitochondrial pellet (suspended in 50  $\mu$ l water) and the supernatant the protease inhibitors chymostatin, antipain, leupeptin (final concentration 0.2 mg ml<sup>-1</sup> each), and aprotinin (0.55 mg ml<sup>-1</sup>) were added. Subsequently, 10% SDS was added to a final concentration of 4%. Both samples were treated for 4 min at 95°C and then diluted 40-fold with Triton buffer (1% Triton X-100, 150 mM sodium chloride, 5 mM EDTA, 50 mM Tris chloride, pH 7.5) to 0.1% SDS. The particulate material was removed by centrifugation at 45,000 rpm (140,000  $\times$   $g_{av}$ ) for 1 h at 15°C, and the supernatant was mixed with 50 to 100  $\mu$ l anti-mAspAT antiserum for 14 to 16 h at 4°C. After incubation, 100-200  $\mu$ l of a 10% (w/v) suspension of Staphylococcus aureus were added and the incubation continued for 1 h at room temperature. After two washings with Triton buffer containing 0.1% SDS and 1 mg ml<sup>-1</sup> methionine the Staphylococcus-antibody-antigen complexes were dissociated in 3 to 5% SDS for 4 min at 95°C and the immunoprecipitation was repeated. The final pellet was dissolved in sample buffer for SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

For processing assays, the labeled translation products were incubated with the supernatant (15,000  $\times$   $g_{max}$ , 10 min, 4°C) of a hypotonic mitochondrial extract (Böhni et al., 1980) as described above for the import assay. After incubation, the total mixture was dissociated in SDS and subjected to immunoprecipitation.

## 2.8 Immunoprecipitation under Nondenaturing Conditions

For immunoprecipitation under nondenaturing conditions the protease inhibitors chymostatin, antipain, leupeptin, aprotinin (final concentration as in the case of immunoprecipitation under denaturing conditions) and phenylmethanesulfonyl fluoride (1 mM) were added to the labeled reticulocyte lysate. When specified, 1/19 volume of 20% Triton X-100 in 10 mM Tris chloride, pH 7.5, was added to the mixture and incubated for 15 min at 4°C. The sample (80-90  $\mu$ l) was mixed with incubation buffer (150 mM potassium chloride, 10 mM Tris chloride, pH 7.5), or with the same buffer plus 1% Triton X-100; final volume 1 ml). The incubation buffer contained 10  $\mu$ l of anti-mAspAT antiserum which had been incubated with saturating amounts of mAspAT (5  $\mu$ g) for 15 min at room temperature just before addition of the sample. Incubation was for 14 to 16 h at 4°C. After incubation 30  $\mu$ l of a 10% (w/v) suspension of Staphylococcus aureus was added and the incubation continued for 1 h at room temperature. After five washings with 150 mM sodium chloride, 5 mM EDTA, 50 mM Tris chloride, pH 7.5, or with Triton buffer the Staphylococcus-immunocomplexes were dissociated in 4% SDS for 4 min at 95°C and the immunoprecipitation was repeated under denaturing conditions.

## 2.9 Miscellaneous

Mitochondrial AspAT was isolated from chicken heart as described (Gehring et al., 1977). AspAT activity was measured by the coupled assay with malate dehydrogenase (Birchmeier et al., 1973). Mitochondrial protein concentration was estimated assuming a specific mAspAT content of 4.6  $\mu$ g per mg mitochondrial protein (Behra et al., 1982) and a specific activity of mAspAT of 220 U mg<sup>-1</sup> at 25°C. The reticulocyte lysate was prepared and pre-

incubated with nuclease from Staphylococcus aureus as described by Pelham and Jackson (1976). Antisera against mAspAT were obtained as described (Sonderegger et al., 1982) except that an equimolar mixture of native and denatured mAspAT was used as immunogen. SDS-polyacrylamide gel electrophoresis was performed in 10% or 12.5% slab gels (Laemmli, 1970). For fluorography of the dried gels in Enlightning the recommendations of the producer (New England Nuclear) were followed. Radioactivity in protein bands on SDS-polyacrylamide gels was determined by counting the radioactivity eluted from cut-out gel pieces (Sonderegger et al., 1982). Peroxidase activity was measured according to Pütter (1974). Labeling of SDS-denatured mAspAT with N-2,3-[ $^{14}\text{C}$ ]ethylmaleimide (Amersham) was performed as described elsewhere (Gehring and Christen, 1978).  $^{14}\text{C}$ -labeled  $M_r$  marker proteins and [ $^{35}\text{S}$ ]methionine ( $\sim 1000 \text{ Ci/mmol}$ ) were from New England Nuclear; o-phenanthroline from Fluka; bathophenanthroline disulfonate disodium salt from Serva. Bovine trypsin (TPCK-treated,  $3.5 \text{ U mg}^{-1}$ ) was from Merck. The protease inhibitors chymostatin, antipain and leupeptin were from the Peptide Institute, Osaka. Aprotinin was from Bayer. Glutaraldehyde-fixed Staphylococcus aureus cells were prepared as described elsewhere (Kessler, 1975).

### 3. RESULTS

#### 3.1 Sedimentation Behavior of pre-mAspAT in a Reticulocyte Lysate

Studies on the intracellular location of pre-mAspAT in chicken embryo fibroblasts pulsed with [ $^{35}$ S]methionine in the presence of CCCP showed that the bulk of pre-mAspAT was present in the postribosomal supernatant, the distribution of pre-mAspAT corresponding with that of cAspAT (Jaussi et al., 1982). In order to examine whether pre-mAspAT is also released from the ribosomes into the cytosol fraction of a heterologous cell-free protein synthesizing system the following experiment was performed. Free polysomes were used to direct protein synthesis in a reticulocyte lysate or in a postribosomal supernatant of a reticulocyte lysate. At the end of the incubation both samples were centrifuged. The two pellet fractions and the two postribosomal supernatants were analyzed by immunoprecipitation first with anti-mAspAT antiserum then with anti-cAspAT antiserum. The distribution of pre-mAspAT proved independent of whether it had been synthesized in the postribosomal supernatant of a reticulocyte lysate or in the total reticulocyte lysate (Fig. 1). In both cases the bulk of the precursor was found in the pellets. In contrast, the major part of cAspAT was found in the supernatant fraction both after synthesis in the postribosomal supernatant and after synthesis in the total reticulocyte lysate. The quantitative evaluation of the distribution of pre-mAspAT and cAspAT is given in Table 1. The amount of pre-mAspAT found in the two pellet fractions corresponded to about 85% of the total precursor and the amount of cAspAT in the two supernatants corresponded to 65% of the total. Clearly, pre-mAspAT and cAspAT exhibit a different sedimentation behavior. These results agree with the previous



**Fig. 1** Sedimentation behavior of pre-mAspAT and cAspAT in a postribosomal supernatant of a reticulocyte lysate and in the total reticulocyte lysate. A postribosomal supernatant of a reticulocyte lysate (PRS) or the total reticulocyte lysate (TRL) were programmed with chicken liver free polysomes for 40 min in the presence of [ $^{35}$ S]methionine. The incubation was stopped by chilling and both samples were centrifuged at  $190,000 \times g$  for 1 h at  $2^\circ\text{C}$ . The supernatants (S) and the pellets (P) were dissociated in SDS and analyzed for mAspAT and cAspAT by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. The fluorogram is shown. The position of pre-mAspAT (pm) is indicated and corresponds to an  $M_r$  of 47,000. The molecular weight markers ( $M_r$ ) are  $^{14}\text{C}$ -labeled cAspAT (c,  $M_r = 46,000$ ) and mAspAT (m,  $M_r = 45,000$ ).

TABLE I Quantitative evaluation of the sedimentation behavior of pre-mAspAT and cAspAT in a postribosomal supernatant of a reticulocyte lysate and in the total reticulocyte lysate. The pre-mAspAT and cAspAT bands shown in Fig. 1 were cut out from the gel and the radioactivity was counted as described (Sonderegger et al., 1982). S, supernatant; P, pellet.

|                       | Postribosomal Supernatant of Reticulocyte Lysate |      | Total Reticulocyte Lysate |      |
|-----------------------|--------------------------------------------------|------|---------------------------|------|
|                       | S                                                | P    | S                         | P    |
| pre-mAspAT band (cpm) | 410                                              | 2250 | 340                       | 2150 |
| cAspAT band (cpm)     | 950                                              | 510  | 900                       | 500  |

estimate (Sonderegger et al., 1982) that at least 2/3 of the total pre-mAspAT sedimented with the insoluble material of the reticulocyte lysate at 140,000 x g. It was noted that pre-mAspAT accounted for about 0.03% of the total protein synthesized in a reticulocyte lysate programmed with free polysomes, whereas in a previous study (Sonderegger et al., 1980) pre-mAspAT amounted to only 0.01% of the total synthesized protein present in the postribosomal supernatant of a reticulocyte lysate programmed with mRNA.

The distribution of pre-mAspAT indicated that the precursor was not associated with endogenous sedimentable particles of the reticulocyte lysate but rather with the free polysomes directing protein synthesis. This possibility was examined with the

following experiment. A translation mixture was centrifuged on an isokinetic sucrose gradient. The sedimentation profile (Fig. 2A) indicated that after an incubation of 40 min a large part of the ribosomes were still present as small polysomes. Pre-mAspAT was immunoprecipitated from the pooled fractions of the sucrose gradient. Only about 20% of the total synthesized precursor (Fig. 2B) but 68% of the trichloroacetic acid-precipitable material were found in the upper part of the gradient. This amount of pre-mAspAT agrees with that detected in the post-ribosomal supernatant after centrifugation of a translation mixture (Table 1). The bulk of pre-mAspAT was found still associated with small polysomes (Fig. 2B, lane 3) and with monosomes and 60S ribosomal subunits (Fig. 2B, lane 4). The immunoreactive material with molecular weights lower than that of pre-mAspAT probably represents nascent polypeptide chains of the precursor (Fig. 2B, lanes 2, 3, and 4). Prolonged incubation of the polysomes with the reticulocyte lysate for 60 min did not further increase the amount of trichloroacetic acid-precipitable radioactive material and also did not change the sedimentation profile of the translation mixture. Apparently, polypeptide chain termination and/or release become limiting during the incubation (see Discussion, 4.1).

### 3.2 Proteolytic Susceptibility of pre-mAspAT

During the studies on the biosynthesis of AspAT in chicken embryo fibroblasts it was noted that pre-mAspAT was exceptionally susceptible toward exogenous proteases (Jaussi et al., 1982). The precursor in contrast to the mature enzyme was completely degraded by treatment with  $80 \mu\text{g ml}^{-1}$  trypsin for 20 min at  $37^\circ\text{C}$ . In the present study, the precursor synthesized in a cell-free system proved to be equally sensitive toward proteolytic degra-

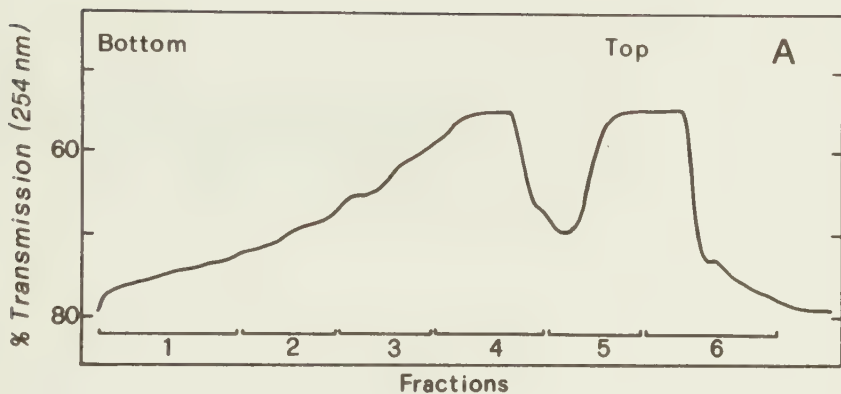
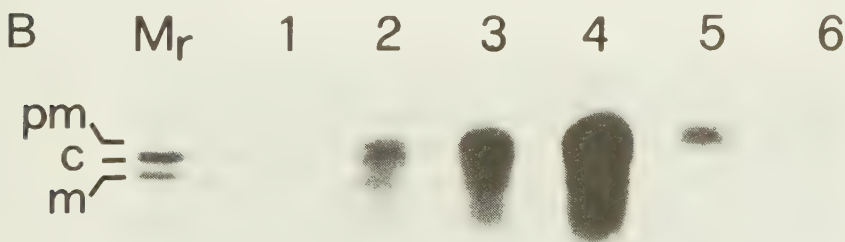


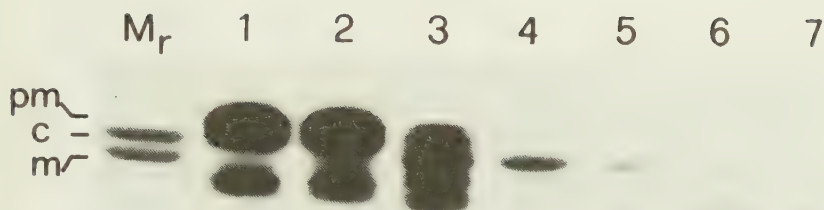
Fig. 2 Distribution of pre-mAspAT after centrifugation of a translation mixture through an isokinetic sucrose gradient.

A: Sedimentation profile: After protein synthesis in a reticulocyte lysate programmed with free polysomes in the presence of [ $^{35}$ S]-methionine, 300  $\mu$ l of the translation mixture were centrifuged through an isokinetic sucrose gradient as described in the "Experimental Procedure" section except that centrifugation was for 2 h. The bars indicate the fractions pooled.



B: Analysis of the pooled fractions by immunoprecipitation: Lanes 1-6 pre-mAspAT immunoprecipitated with anti-mAspAT antiserum from the corresponding pools in Fig 2A. Marker proteins ( $M_r$ ) are the same as in Fig.1; pm denotes the position of pre-mAspAT.

dation. A reticulocyte lysate containing labeled precursor was incubated at 4°C for 20 min with increasing amounts of trypsin (Fig. 3, lanes 2 to 7). Before the addition of trypsin mature mAspAT was added to each sample. Degradation of pre-mAspAT was already noticed at the lowest trypsin concentration tested ( $5 \mu\text{g ml}^{-1}$ ) and was complete at a trypsin concentration of  $10 \mu\text{g ml}^{-1}$ . In contrast, mAspAT was not degraded even at the highest trypsin concentrations. The pronounced resistance of mAspAT toward trypsin (Sandmeier and Christen, 1980) ruled out the classical "protection assay" in import studies. The "protection assay" exploits the fact that the uptake into mitochondria renders the imported polypeptides inaccessible and, hence, resistant to proteases added. Of course, this assay is unsuitable to monitor the translocation of the polypeptide through the mitochondrial membrane if processing of the precursor produces a mature protein that is protease-resistant per se. Therefore, the effect of several proteases other than trypsin on mAspAT was examined. Crotalus protease, Staphylococcus aureus protease, proteinase K, Carlsberg Subtilisin and chymotrypsin all proved to be not more efficient than trypsin. Use of proteases at higher concentrations was not possible because the conditions of proteolysis used in the "protection assay" should not damage the mitochondria. These conditions correspond for example in the case of trypsin or proteinase K (Gasser et al., 1982b; Schleyer et al., 1982) to protease concentrations of 100 to  $200 \mu\text{g ml}^{-1}$  and incubation at 4°C for 30 min. Thus, the "protection assay" with the proteases tested was not possible in the case of mAspAT. Instead, the translocation of pre-mAspAT was assessed by examining the effect of inhibitors of import and processing (Gasser et al., 1982b) on the conversion of pre-mAspAT to mAspAT by intact mitochondria or by a mitochondrial extract (see this section, 3.8).



**Fig. 3** Proteolytic susceptibility of pre-mAspAT. After protein synthesis in a reticulocyte lysate 6 samples (60  $\mu$ l each) were incubated with different amounts of trypsin for 20 min at 4°C. Before the addition of trypsin each sample received 2  $\mu$ g of mature mAspAT. Incubation was stopped by the addition of a mixture of chymostatin, antipain, leupeptin (0.35 mg ml<sup>-1</sup>, each) and aprotinin (0.78 mg ml<sup>-1</sup>). All samples were added to 60  $\mu$ l hot 10% SDS/5% 2-mercaptoethanol and heated for 4 min at 95°C. The samples were then lyophilized and taken up in 100  $\mu$ l water. After repeating this procedure thrice the samples were subjected to immunoprecipitation with antiserum against mAspAT and SDS-polyacrylamide gel electrophoresis. The fluorogram is shown. Lane 1, sample incubated as a control with 5  $\mu$ l 1 mM HCl, lanes 2 to 7, samples containing 5, 10, 20, 50, 100 and 300  $\mu$ g ml<sup>-1</sup> of trypsin (added in 5  $\mu$ l), respectively. The denotations pm, m and c are the same as in Fig. 1.

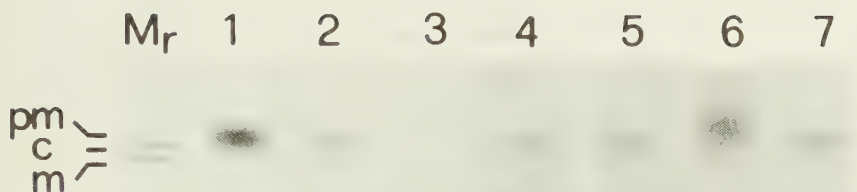
### 3.3 Immunological Characterization of pre-mAspAT

The structures of pre-mAspAT and mAspAT were compared by an immunological approach. Previous studies (Sonderegger et al., 1980) had indicated that the native precursor polypeptide contains epitopes which are present in the native mature protein. In order to determine whether native pre-mAspAT also contains epitopes which are present in denatured mAspAT but are lacking in the native mature protein, immunoprecipitations were performed in a nondenaturing system (see Experimental Procedure, 2.8). An antiserum obtained by immunization with a mixture of native and denatured mAspAT was saturated with native mAspAT;

henceforth, it will be denoted anti-denatured mAspAT antiserum. On incubation of a sample of reticulocyte lysate containing labeled translation products with anti-denatured mAspAT antiserum part of pre-mAspAT was immunoprecipitated (Fig. 4, lane 2). Denatured mAspAT but not native mAspAT completely competed out pre-mAspAT (Fig. 4, lanes 2 and 3). In order to exclude an artifact brought about by Triton X-100, e.g., denaturation of pre-mAspAT, immunoprecipitations were performed without foregoing treatment of the samples with Triton X-100 (see Experimental Procedure, 2.8). The results agreed with those obtained after Triton X-100 treatment (Fig. 4, lanes 5, 6, and 7). The amount of precursor immunoprecipitated under nondenaturing conditions corresponded to about 50% of total pre-mAspAT which was recovered by the usual immunoprecipitation under denaturing conditions (Fig. 4, lane 1). In two other experiments the recovery of precursor under nondenaturing conditions was even 75 and 100%. In these two experiments the immunological competition with native and denatured mAspAT gave the same result as the experiment shown in Fig. 4. Very likely, the incomplete recovery in some of the experiments was due to a nonquantitative immunological extraction.

### 3.4 Aggregation State of in vitro and in vivo Synthesized pre-mAspAT

Both the in vitro and in vivo synthesized precursor of mAspAT were analyzed by high performance gel permeation chromatography. A postribosomal supernatant of a labeled reticulocyte lysate or a labeled postmitochondrial supernatant of chicken embryo fibroblasts pulsed in the presence of CCCP (Jaussi et al., 1982) were applied to the column. The bulk of both in vitro and in vivo synthesized pre-mAspAT was eluted with the void volume



**Fig. 4** Immunological characterization of pre-mAspAT. Following protein synthesis in a reticulocyte lysate, samples of the translation mixture were immunoprecipitated with anti-denatured mAspAT antiserum (see text) (lanes 2 to 7) in the presence of 5  $\mu$ g denatured mAspAT (lanes 3 to 6) or of 5  $\mu$ g native mAspAT (lanes 4 to 7). Before incubation with antiserum, Triton X-100 was added to three samples (lanes 2, 3, 4) to a final concentration of 1% (w/v). As a control, one sample was immediately denatured after protein synthesis and immunoprecipitated under denaturing conditions (lane 1). The denotations pm, m, and c are the same as in Fig. 1.

(Fig. 5A and 5B) corresponding to a minimal  $M_r$  of 300,000. Apparently, after synthesis pre-mAspAT becomes part of a high molecular weight complex. In the case of pre-mAspAT from the reticulocyte lysate a minor fraction was eluted in a broad peak corresponding to an apparent  $M_r$  of 75,000 (Fig. 5A). This elution volume corresponds with that of mAspAT (Fig. 5C) which for unknown reasons does not elute corresponding to its  $M_r$  of 90,000. In the case of the in vivo synthesized product, faint bands of pre-mAspAT could be detected corresponding to an  $M_r$  of  $\sim$  40,000 (Fig. 5B) suggestive of a small pool of monomer.

In these experiments (and in some others, see e.g., Fig. 3) pre-mAspAT appeared as a double band, the additional band having a slightly lower molecular weight. The relation of this band to mAspAT was first indicated by the fact that it was competed out by mAspAT but not by cAspAT, and further confirmed by peptide mapping. The immunoreactive band was analyzed by chemical cleavage of the protein in the gel slice and re-electrophoresis of

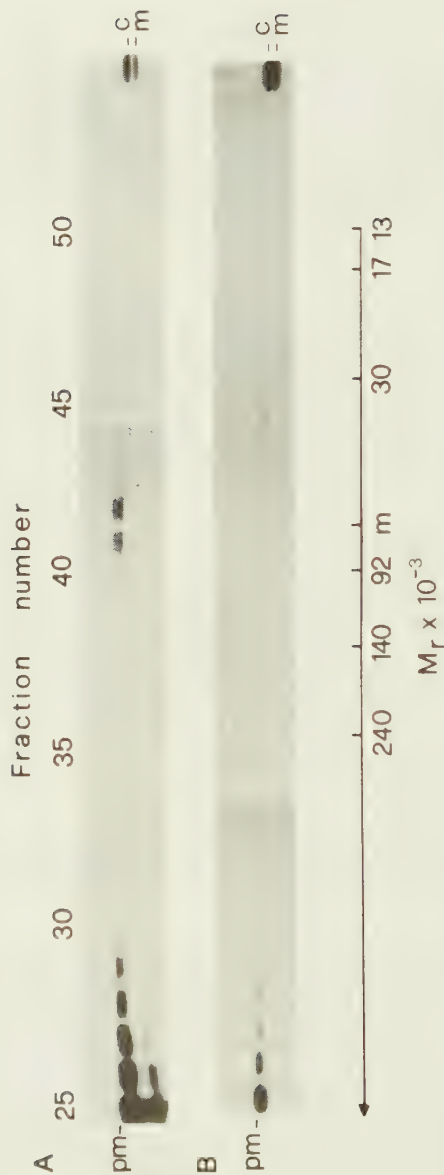


Fig. 5A,B Gel permeation chromatography of pre-mAspAT. (A) The postribosomal supernatant of a reticulocyte lysate containing pre-mAspAT labeled with [ $^{35}$ S]methionine or (B) the postmitochondrial supernatant of chicken embryo fibroblasts pulsed with [ $^{35}$ S]methionine in the presence of 20  $\mu$ M CCCP were applied to a gel permeation chromatography column. To both samples 4  $\mu$ g of mAspAT were added as marker. The eluted fractions were subjected to immunoprecipitation, SDS-polyacrylamide gel electrophoresis and fluorography. The  $M_r$  values indicated are from the calibration run of Fig. 5C. The denotation "m" indicates the elution volume of marker mAspAT as visualized by protein staining. It corresponds to an  $M_r$  value of 75,000. The same elution volume of mAspAT was found in the calibration run.

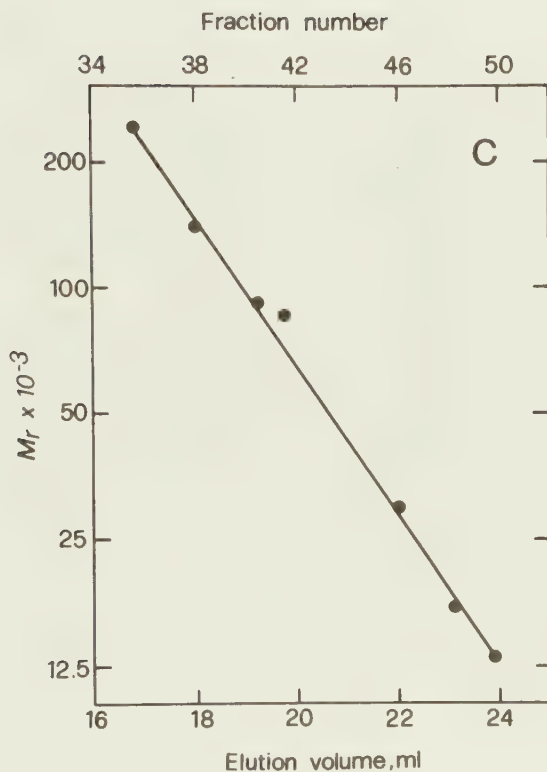
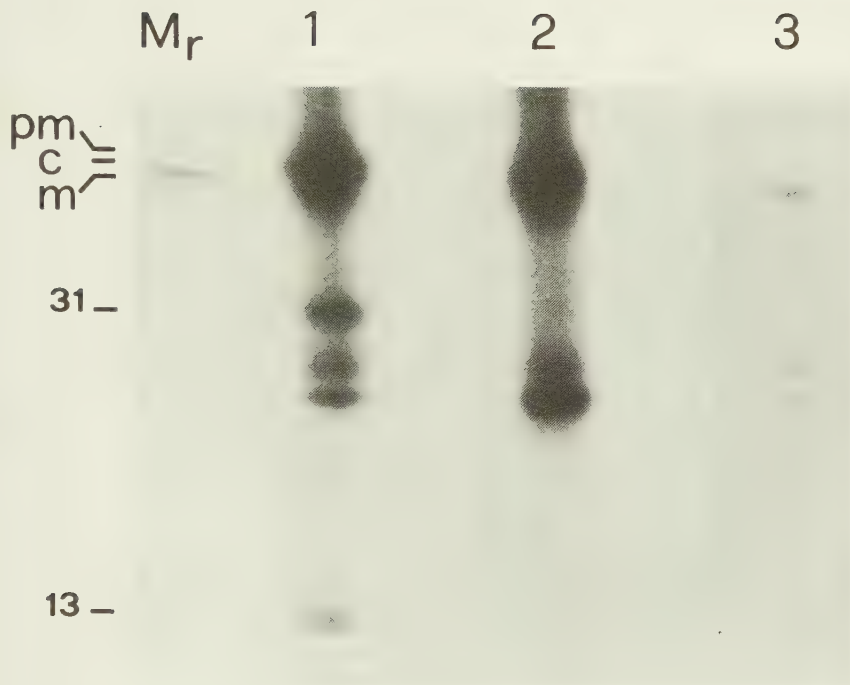


Fig. 5C Calibration of the gel permeation chromatography column. The following  $M_r$  markers were applied to the column: Catalase (240,000); lactate dehydrogenase (140,000); cAspAT (92,000); mAspAT (90,000; however, its elution volume corresponds to an  $M_r$  of 75,000); carboanhydrase (30,000); myoglobin (17,000); cytochrome c (13,000).

the fragments (Sonderregger et al., 1982a). The banding pattern of the fragments (Fig. 6, lane 2) was very similar to that of the cleaved pre-mAspAT and that of the cleaved mature mAspAT (Fig. 6, lanes 1 and 3, respectively). The immunoreactive band probably represents a proteolytic product of the precursor. This product does not represent a maturation intermediate of pre-mAspAT because its appearance in the reticulocyte lysate was sporadic and independent of the presence of mitochondria.

The aggregation state of both in vitro and in vivo synthesized pre-mAspAT was also studied by zone velocity centrifugation in sucrose gradients. About half of pre-mAspAT from a reticulocyte lysate sedimented in an  $M_r$  range around 85,000 and the other half in the high  $M_r$  range of the gradient ( $M_r > 240,000$ ) suggesting that under the conditions used pre-mAspAT exists as a dimer and as part of a higher  $M_r$  aggregate (Fig. 7A and 7D). The distribution of pre-mAspAT between the two forms was not constant. In another experiment the ratio between the two peaks changed from 1:1 to 4:1 in favor of the putative dimeric form.

On centrifugation of a postmitochondrial supernatant from CCCP-treated chicken embryo fibroblasts, the precursor sedimented diffusely in an  $M_r$  range from  $\sim 40,000$  to  $> 240,000$  corresponding to 1 to  $> 6$  times its  $M_r$  in SDS containing gels (Fig. 7B and 7D). The  $M_r$  distribution of newly processed mAspAT was examined in the same way. A homogenate of pulsed chicken embryo fibroblasts was sonicated in order to release the labeled enzyme from the mitochondria. After centrifugation, mAspAT was detected as a distinct peak in the 85,000 region of the gradient (Fig. 7C and 7D) indicating that after transport into the mitochondria newly processed mAspAT assembles to dimers. Mature endogenous unlabeled mAspAT was immunoprecipitated from the same fractions as newly processed mAspAT (not shown).

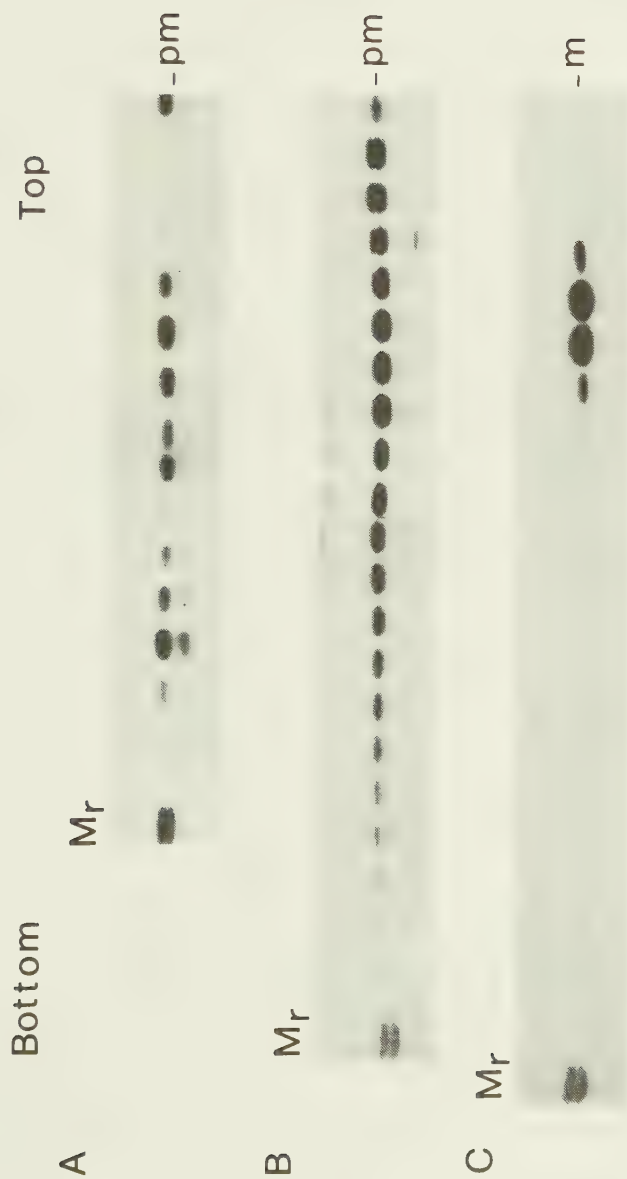


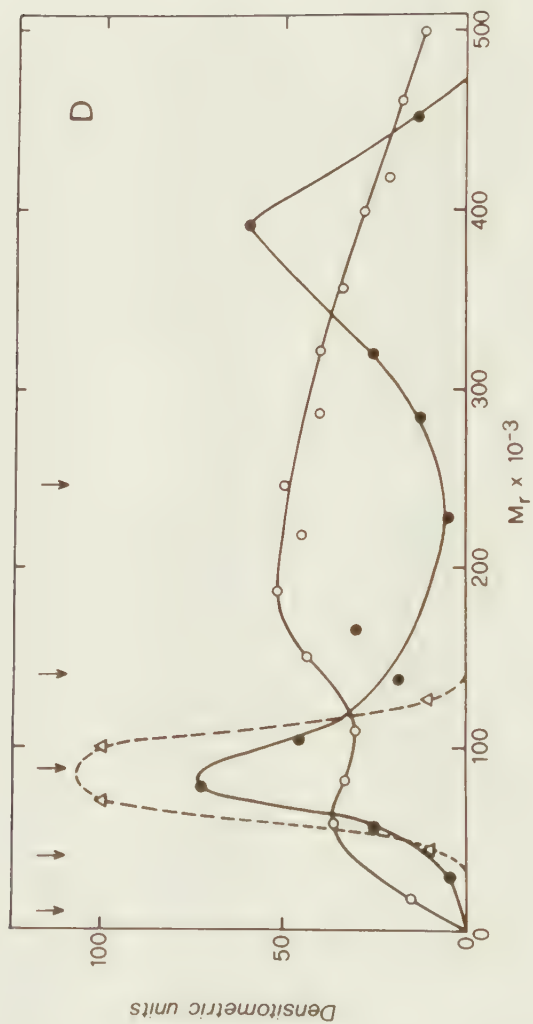
**Fig. 6** Peptide maps of pre-mAspAT, the related immunoreactive band, and mAspAT. The pre-mAspAT band (lane 1) and the related immunoreactive band (lane 2) of the gel of Fig. 5A were cut out and incubated in 75% (v/v) formic acid at 37°C for 24 h (Sonderegger et al., 1982a). The fragments were then re-electrophoresed on a 12.5% SDS-polyacrylamid gel. Lane 3 represents cleaved mature mAspAT which had been labeled with [<sup>14</sup>C]N-ethylmaleimide. The position of pre-mAspAT (pm) is indicated and corresponds to an M<sub>r</sub> of 47,000. The <sup>14</sup>C-labeled marker proteins are cAspAT (c), M<sub>r</sub> = 46,000; mAspAT (m), M<sub>r</sub> = 45,000; carbonic anhydrase, M<sub>r</sub> = 31,000; cytochrome c, M<sub>r</sub> = 13,000.

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Fig. 7A,B,C Sucrose gradient centrifugation of pre-mAspAT and newly processed mAspAT. (A) The postribosomal supernatant of a reticulocyte lysate or (B) the postmitochondrial supernatant of CCCP-treated chicken embryo fibroblasts, each containing labeled pre-mAspAT, or (C) the sonicated homogenate of untreated chicken embryo fibroblasts containing newly processed mAspAT were centrifuged on linear sucrose gradients for 20 h. (For details on the preparation of the samples A, B and C, see Experimental Procedure, 2.4). After centrifugation, 5 fractions of 1 ml each corresponding to the bottom part of the gradient and 12 fractions of 0.5 ml each corresponding to the top part of the gradient (A) or 22 fractions of 0.5 ml each (B and C) were collected and analyzed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. Marker proteins ( $M_r$ ) are the same as in Fig. 1. The positions of pre-mAspAT (pm) and mAspAT (m) are indicated.

Fig. 7D Quantitative evaluation of the sucrose gradient centrifugation of pre-mAspAT and newly processed mAspAT. The bands of the fluorograms of Figs. 7A, B and C were scanned densitometrically. The readings were plotted against the corresponding  $M_r$  values. ●, pre-mAspAT synthesized in a reticulocyte lysate; ○, pre-mAspAT synthesized in chicken embryo fibroblasts; △, newly processed mAspAT from chicken embryo fibroblasts. Five downward arrows show the positions of the  $M_r$  markers: Cytochrome c (13,000), peroxidase (40,000), mAspAT (90,000), lactate dehydrogenase (140,000), and catalase (240,000).  $M_r$  values > 240,000 were estimated by extrapolation. The marker proteins, dissolved in 150 mM potassium chloride, 10 mM Tris chloride, pH 7.4, were centrifuged in separate tubes in the same run. Their positions were determined by the absorbance of the eluted fractions at 280 nm and when plotted against the  $M_r$  values gave straight lines. If mitochondrial AspAT and peroxidase were added to a postribosomal supernatant from a reticulocyte lysate or to a postmitochondrial supernatant from homogenized chicken embryo fibroblasts and were centrifuged in parallel to the  $M_r$  marker proteins, their positions in the gradients were found to be unchanged as determined by measuring their enzymic activities.



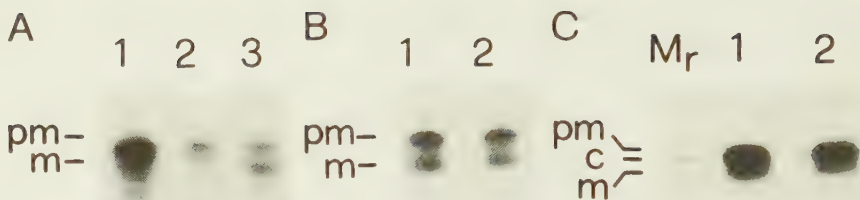


### 3.5 In vitro Import of pre-mAspAT into Isolated Chicken Heart Mitochondria

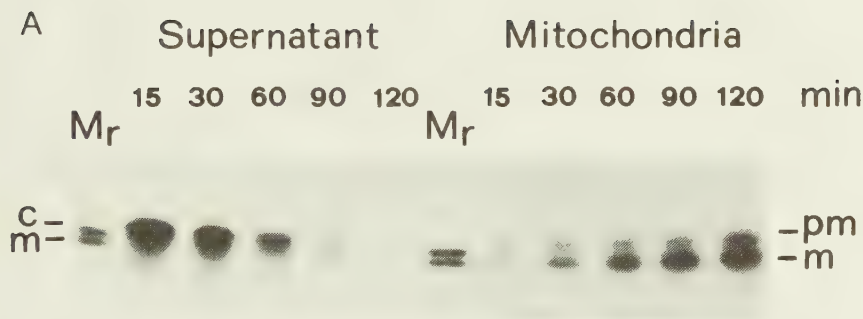
A rabbit reticulocyte lysate was programmed with chicken liver polysomes in the presence of [ $^{35}\text{S}$ ]methionine. After stopping translation, a sample of the lysate was incubated with isolated mitochondria. The incubation mixture was then centrifuged, and the supernatant and the mitochondrial pellet were analyzed separately for labeled pre-mAspAT and mature mAspAT (Fig. 8A). The supernatant contained only precursor, whereas the mitochondrial pellet contained both precursor and mature mAspAT. Apparently, part of the precursor had been merely bound to the mitochondria while part of it had been taken up by the organelles and had been processed to the mature protein. Exogenous mature mAspAT did not compete with the precursor for uptake into mitochondria (Fig. 8B). In order to exclude the possibility that the uptake of labeled protein into mitochondria occurred cotranslationally, protein synthesis in the translation mixture was stopped by cycloheximide and a postribosomal supernatant was prepared. Consistent with a posttranslational uptake mechanism, incubation of this postribosomal supernatant with mitochondria yielded mature mAspAT (Fig. 8C).

### 3.6 Time Dependence of Import

The conversion of precursor to mature enzyme is a time-dependent process. On incubation of a labeled translation mixture with exogenous mitochondria the precursor in the supernatant decreased concomitantly with an increase of mature mAspAT in the mitochondria during the first 90 min of incubation (Fig. 9A). No mature enzyme was detected in the supernatant indicating that the mitochondria remained intact during the incubation.

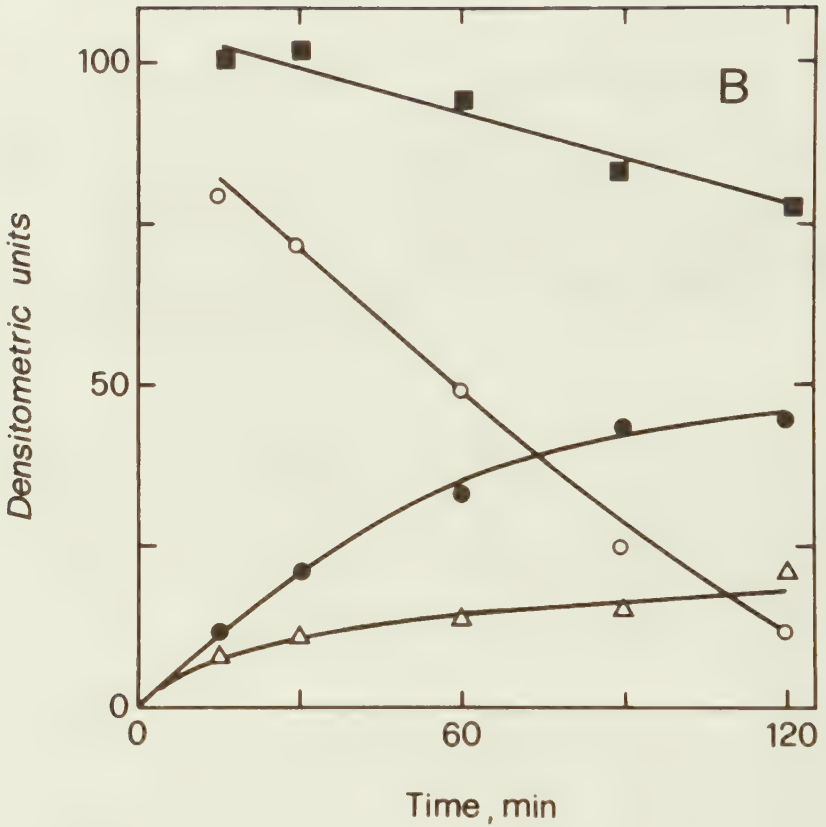


**Fig. 8A,B,C** In vitro import of pre-mAspAT into isolated chicken heart mitochondria. (A) A reticulocyte lysate programmed with chicken liver free polysomes and labeled with [ $^{35}\text{S}$ ]methionine ( $1 \text{ mCi ml}^{-1}$ ) was incubated with or without chicken heart mitochondria at  $29^\circ\text{C}$  for 60 min as described in Experimental Procedure, 2.7. The incubation was stopped by chilling and the lysate containing the mitochondria was fractionated into mitochondrial pellet and supernatant. All fractions were analyzed for mAspAT by immunoprecipitation, SDS-polyacrylamide gel electrophoresis and fluorography: Total reticulocyte lysate incubated without mitochondria (lane 1); supernatant (lane 2), and mitochondrial pellet (lane 3) of lysate incubated with mitochondria. The band of pre-mAspAT (pm) corresponds to an  $M_r$  of 47,000; mature mAspAT (m) has an  $M_r$  of 45,000. (B) Mature mAspAT ( $0.3 \mu\text{M}$ , subunit concentration) was added to a reticulocyte lysate labeled with [ $^{35}\text{S}$ ]methionine. The sample was incubated with mitochondria for 60 min as described for experiment A. The mitochondrial pellets are shown. Lane 1, mAspAT added; lane 2, control without added mAspAT. (C) After read-out translation two samples of the reticulocyte lysate were withdrawn, one received  $0.2 \text{ mM}$  cycloheximide, and from both a postribosomal supernatant was prepared. Mitochondria were added to both supernatants as described for experiment A. The mitochondrial pellets are shown. Lane 1, no cycloheximide added; lane 2, with cycloheximide;  $M_r$ ,  $^{14}\text{C}$ -labeled marker proteins: cAspAT (c,  $M_r = 46,000$ ), mAspAT (m).



**Fig. 9A** Time dependence of import of pre-mAspAT into mitochondria. Five samples of labeled translation mixture were incubated with exogenous mitochondria and after the indicated periods of time separated by centrifugation into supernatant and mitochondrial fraction and analyzed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis and fluorography. The molecular weight markers ( $M_r$ ) are  $^{14}\text{C}$ -labeled cAspAT (c,  $M_r = 46,000$ ) and mAspAT (m,  $M_r = 45,000$ ). The band of pre-mAspAT (pm) corresponds to an  $M_r$  of 47,000.

A quantitative evaluation of the results is given in Fig. 9B. After an incubation of 120 min about 50% of the total precursor present at 15 min was converted to the mature form. The percentage of pre-mAspAT cosedimenting with mitochondria increased from 10% after 15 min to  $\sim 20\%$  after 120 min. The cosedimented precursor, like the precursor remaining in the supernatant and in contrast to the processed precursor, was completely digested by very low concentrations of trypsin ( $10 \mu\text{g ml}^{-1}$ , 20 min,  $4^\circ\text{C}$ ; experiments not shown); apparently, it was bound to the outer mitochondrial membrane. The total immunoprecipitable material (pre-mAspAT plus mAspAT) after an incubation of 2 h was 75% of the total material immunoprecipitated after 15 min indicating a slow continuous degradation of the precursor.



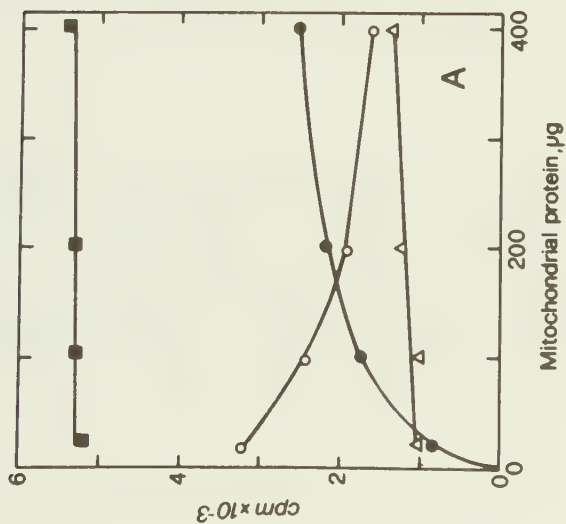
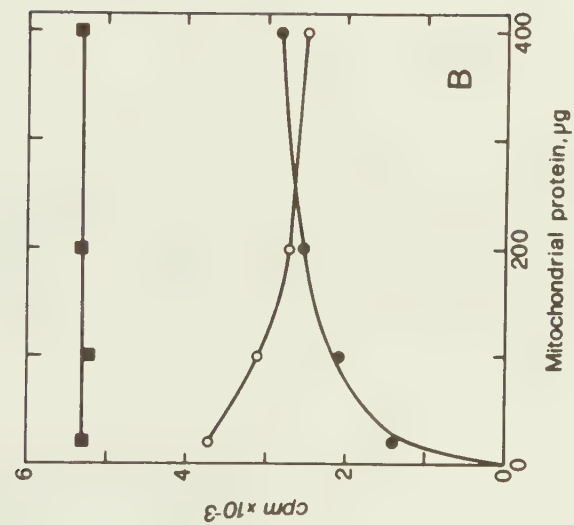
**Fig. 9B** Quantitative evaluation of the time-course of the import of pre-mAspAT. The bands of the fluorograms of Fig. 9A were scanned densitometrically. o, Pre-mAspAT in the supernatant; ●, mAspAT in mitochondria; Δ, pre-mAspAT cosedimenting with mitochondria; ■, sum of total pre-mAspAT plus mAspAT.

### 3.7 Rates of Import and Processing

Incubation of a labeled reticulocyte lysate with increasing amounts of mitochondria or with corresponding increasing amounts of hypotonic mitochondrial extract results in an accelerated generation of mature mAspAT (Figs. 10A and 10B, respectively). In both cases, a maximum value of imported or processed precursor is reached asymptotically at 200 to 400  $\mu\text{g}$  of mitochondrial protein added. This maximum value is 50% of the total precursor and equals the maximum fraction of pre-mAspAT found to be imported in the study of the time dependence of import (Fig. 9). Thus, the maximum percentage of imported or processed pre-mAspAT is limited neither by time nor by the amount of mitochondria or mitochondrial protein added. Addition of unlabeled reticulocyte lysate (nuclease-treated or untreated) in 6-fold excess compared to the labeled reticulocyte lysate present in the import assay, as well as addition of chicken heart cytosol and chicken fibroblast cytosol to the import assay failed to increase the yield of import. Such additions were reported to stimulate the in vitro import of the precursors of ornithine transcarbamylase and of cytochrome  $b_2$  into mitochondria (Miura et al., 1983; Ohta and Schatz, 1984). Addition of  $\text{Zn}^{2+}$  or  $\text{Co}^{2+}$  to the mitochondrial extract (Böhni et al., 1983) did not increase the fraction of processed precursor. Apparently, in the reticulocyte lysate used in these studies only  $\sim 50\%$  of the synthesized precursor are present in a form that can be imported or processed. The same fraction of importable precursor was obtained with different preparations of polysomes. However, with other batches of reticulocyte lysate the fraction was even smaller.

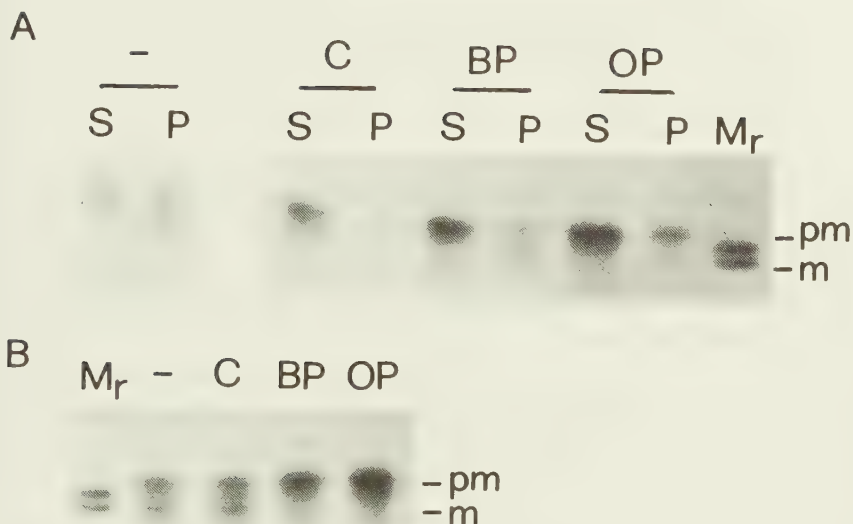
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Fig. 10A,B Rates of import and processing. Four samples of [ $^{35}$ S]methionine labeled translation products were incubated (A) with varying amounts of isolated mitochondria or (B) with corresponding amounts of a supernatant of hypotonically disrupted mitochondria (final volume 85  $\mu$ l; for details, see Experimental Procedure, 2.7). After 90 min at 29°C the supernatant and mitochondrial fractions of the import assays A and the processing assays B were made 4% (w/v) in SDS and analyzed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. The bands corresponding to pre-mAspAT and mAspAT were cut out from the gels and the radioactivity counted as described (Sonderregger et al., 1982). o, pre-mAspAT in the supernatant of import assay A or pre-mAspAT in processing assay B; ●, mAspAT in the mitochondria or processed mAspAT in assay A or B, respectively;  $\Delta$ , pre-mAspAT associated with the mitochondria; ■, sum of total pre-mAspAT plus mAspAT. Prolonged incubation (2 h) of the samples containing 400  $\mu$ g of mitochondrial proteins did not increase the amount of imported or processed mAspAT.



### 3.8 Inhibitors of Import and Processing

In order to show that pre-mAspAT is processed to the mature form only after having entered the matrix of the mitochondrion, a combination of inhibitors of import and processing were applied (Gasser et al., 1982b). Following read-out translation in a reticulocyte lysate the labeled translation mixture received either the uncoupler carbonyl cyanide m-chlorophenylhydrazone or one of the chelating agents o-phenanthroline or bathophenanthroline disulfonate. The samples were then incubated with either intact mitochondria or with a supernatant of hypotonically disrupted mitochondria. After incubation in the presence of CCCP no mature enzyme could be immunoprecipitated from the mitochondria (Fig. 11A) in agreement with the results obtained in vivo (Jaussi et al., 1982). The effect of CCCP was previously shown to be due to depletion of the electrochemical potential across the inner mitochondrial membrane (Gasser et al., 1982a,b; Schleyer et al., 1982; Kolansky et al., 1982). As expected, CCCP had no effect on the appearance of mature enzyme if pre-mAspAT was incubated with a mitochondrial extract (Fig. 11B). Of the two chelating agents tested only the hydrophobic o-phenanthroline but not the charged bathophenanthroline disulfonate inhibited the appearance of mature enzyme in intact mitochondria (Fig. 11A). When tested with the mitochondrial extract both agents inhibited the processing of pre-mAspAT (Fig. 11B).



**Fig. 11** Inhibitors of import and processing. (A) Mitochondria, or (B) a supernatant of hypotonically disrupted mitochondria were preincubated with the indicated inhibitors for 5 min on ice: C, 10  $\mu$ M CCCP; BP, 1 mM bathophenanthroline disulfonate; OP, 2 mM o-phenanthroline; -, no addition. The mitochondria or the mitochondrial extract were then added to the [ $^{35}$ S]methionine labeled reticulocyte lysate previously adjusted to the same inhibitor concentration. Incubation was for 60 min at 29°C. Supernatants (S) and mitochondrial pellets (P) of the import assay suspensions and the processing assay were made 4% (w/v) in SDS and subjected to immunoprecipitation. Marker proteins ( $M_r$ ) are the same as in Fig. 9A.

## 4. DISCUSSION

### 4.1 Structural Characterization of pre-mAspAT

Extensive studies on mitochondrial biogenesis have established that about 90% of all mitochondrial proteins are coded for by nuclear DNA and are synthesized on cytosolic ribosomes. The determination of the subcellular site of synthesis of the primary translation product of the imported mitochondrial proteins was of central importance in elucidating the mechanism by which these proteins become integrated into the mitochondria. The finding that pre-mAspAT (Sonderegger et al., 1982) and other mitochondrial precursor proteins (for a review, see Doonan et al., 1984) are synthesized predominantly on free polysomes implicated that the precursor polypeptides are released after synthesis into the cytosol and indicated a synthesis-independent mechanism of import. Indeed, a pool of pre-mAspAT was found in the cytosol of chicken embryo fibroblasts (see Introduction, 1.2). In this study a structural characterization of pre-mAspAT was attempted. No properties of the precursor had been defined before with the exception of its marked susceptibility to proteolytic degradation (Jaussi et al., 1982).

The first question addressed was whether the precursor synthesized in a cell-free system was released from the ribosomes into the cytosol fraction. Protein synthesis in a reticulocyte lysate programmed by free polysomes was found to stop after an incubation of about 30 min. On prolonged incubation of the run-off system (Sonderegger et al., 1982) 65% of the total trichloroacetic acid-precipitable material and 65% of the total synthesized cAspAT were released from the ribosomes into the postribosomal fraction. In contrast, only 15-20% of the total synthe-

sized pre-mAspAT were found in the postribosomal supernatant. The bulk of the precursor was found to sediment with monosomes and small polysomes.

Two reasons may explain this distribution of the precursor in the reticulocyte lysate. First, part of the sedimentable precursor, as well as the sedimentable cAspAT and the sedimentable trichloroacetic acid-precipitable material may exist as peptidyl-tRNA-monomosome complexes. This assumption was supported by the analysis of translation mixtures that had been incubated for 60 min and subsequently had been adjusted to 0.3 M potassium chloride on sucrose gradients containing 0.3 M potassium chloride and 3 mM magnesium acetate. Under these conditions, free 80 S monosomes dissociate into subunits whereas mRNA-bound monosomes remain stable (Falvey and Staehelin, 1970). In this experiment, about 50% of the monosomes remained stable and carried labeled polypeptide chains, presumably as peptidyl-tRNA, suggesting that during incubation of the translation mixture polypeptide termination and/or release come to a stop. Secondly, the distribution of the precursor may reflect its exceptionally basic character. The mature enzyme has an isoelectric point of 9.3 (Christen et al., 1985) and the N-terminal extension of pre-mAspAT is expected to increase the total content of basic residues in the protein (see Introduction, 1.1). It is conceivable that negatively charged constituents of the reticulocyte lysate, i.e., ribonucleic acids, interact with the highly positively charged precursor protein (see below).

Interactions of this kind were suggested by experiments (not shown) on the isoelectric point of pre-mAspAT. On chromatofocusing (essentially as described by Endo et al., 1985, except that the starting buffer was adjusted to pH 11), the precursor both in a postribosomal supernatant of a reticulocyte lysate and in

a postmitochondrial supernatant of a homogenate from chicken embryo fibroblasts eluted without a clear maximum in the range pH 9.0 - 7.5, whereas mAspAT (apo form) eluted as a sharp peak at pH 9.7. Similarly, the precursor of rat liver mAspAT was reported having a lower apparent isoelectric point (pH 7) than the mature enzyme (pH 8.5 - 8.8) (Kamisaki et al., 1982). Although it cannot be excluded that the N-terminal prepiece of pre-mAspAT contains indeed acidic amino acid residues, it is probable that the apparent low isoelectric point is due to interactions between the precursor and ribonucleic acids present in the post-ribosomal supernatant of the reticulocyte lysate. Similar interactions between the precursor and ribonucleic acid components of the ribosomes may be responsible for the sedimentation behavior of pre-mAspAT.

The experiments on import of pre-mAspAT indicate that its appearance in the postribosomal supernatant is not inhibited but only retarded. The yield of import of pre-mAspAT was found to be 50% of the total precursor implying that during incubation of the total reticulocyte with mitochondria part of the sedimentable precursor became available for import.

Differences in the sensitivity to proteolytic degradation had given the first indication of a difference in conformation between pre-mAspAT and mAspAT (Jaussi et al., 1982). In this study, a similar experiment confirmed the high susceptibility to trypsin in the case of pre-mAspAT synthesized in a reticulocyte lysate and the resistance of mAspAT. On conversion of the precursor to the mature protein by a mitochondrial extract, mAspAT became resistant to treatment with trypsin. Apparently, the cleavage of the N-terminal prepiece from the precursor suffices to induce resistance toward trypsin. In addition to pre-mAspAT and mAspAT differences in proteolytic susceptibility between pre-

cursor and mature mitochondrial protein have been reported in only one other instance. Porin, an integral protein of the outer mitochondrial membrane is resistant to trypsin even after solubilization with cholate. In contrast, its primary translation product which is synthesized without an additional N-terminal sequence is sensitive to trypsin. Thus, in the case of porin the integration into the mitochondrial outer membrane renders the protein trypsin-resistant (Gasser and Schatz, 1983).

Pre-mAspAT was further characterized by an immunological approach. The precursor was found to react with an anti-denatured mAspAT antiserum; the binding was competed with denatured but not with native mAspAT. However, the differences in recovery of pre-mAspAT by the anti-denatured antiserum in different experiments (see Results, 3.3) suggested that the immunoprecipitations under nondenaturing conditions might not have been quantitative. Thus, the possibility that only denatured precursor reacted with the anti-denatured antiserum cannot be excluded, but seems unlikely on the basis of the following experimental evidence. In one out of a total of three experiments pre-mAspAT was immunoprecipitated quantitatively from the translation mixture. Denatured mAspAT fully competed out the precursor also in this case whereas native mAspAT was without effect. The experimental conditions for immunoprecipitation with anti-denatured mAspAT antiserum corresponded with the conditions for the import assays except for the presence of Triton X-100 and for the 8-fold higher dilution of the translation mixture. Omitting Triton X-100 did not alter the immunoreactivity of the precursor. In the import assays about 50% of the total precursor were found to be imported into mitochondria indicating that at least this fraction of the precursor is present in native form. Apparently, at least part of pre-mAspAT immunoprecipitated by anti-denatured mAspAT antiserum exists in importable, i.e., native form. Thus, the native precursor

sor contains epitopes that are present in denatured mAspAT and are lacking in the native protein. These results again indicate that a conformational change occurs when pre-mAspAT is converted to the mature protein. Immunological properties analogous to that of pre-mAspAT were found for the precursor of mitochondrial malate dehydrogenase (Chien and Freeman, 1983). In this case, antibodies specific for SDS-denatured malate dehydrogenase reacted with the precursor but not with the native mature protein. The results were also interpreted as to reflect a conformational change of the precursor upon its processing by mitochondria.

Studies on the aggregation state of the precursor showed that pre-mAspAT both in a reticulocyte lysate and in a postmitochondrial supernatant from chicken embryo fibroblasts is not a homogeneous species. Centrifugation and gel filtration experiments indicate that pre-mAspAT exists as high ( $> 300,000$ ) and low  $M_r$  complexes. With both methods the apparent  $M_r$  of the low  $M_r$  form of the precursor corresponds with the  $M_r$  determined for mature mAspAT. Thus, the low  $M_r$  material may represent a dimeric form of pre-mAspAT although an association of the precursor with other proteins cannot be excluded. The aggregation state of the precursor seems to be influenced by the experimental conditions. Gel filtration, which was performed in the short time of 12 min (elution time of high  $M_r$  aggregates) at  $20^\circ\text{C}$ , favored the detection of the high  $M_r$  aggregates while after centrifugation, for 20 h at  $2^\circ\text{C}$ , the ratio between high and low  $M_r$  material was shifted towards the latter.

For several other mitochondrial precursor proteins  $M_r$  values ranging from monomer up to 500,000 have been found. Each protein under a given set of conditions yielded a characteristic aggregation profile. On gel filtration analysis the bulk of the

precursors of the  $\beta$ -subunit of yeast  $F_1$ -ATPase (Hay et al., 1984), of rat liver ornithine transcarbamylase (Miura et al., 1981), of Neurospora ATP/ADP carrier (Zimmermann and Neupert, 1980) and of rat liver adenine nucleotide carrier (Chien and Freeman, 1983) appeared as high  $M_r$  aggregates. On centrifugation the precursors of rat liver ornithine transcarbamylase (Miura et al., 1981) and carbamoylphosphate synthetase (Miura et al., 1981) as well as the precursor of subunit 9 of Neurospora ATPase (Schmidt et al., 1983) showed a broad  $M_r$  profile ranging from monomer up to high  $M_r$  aggregates. For the precursor of rat liver mAspAT (Sakakibara et al., 1983) and malate dehydrogenase (Chien and Freeman, 1983)  $M_r$  values only slightly larger than for the mature dimeric form of the corresponding enzyme have been determined by gel filtration. The rather extensive variation in the aggregation profile of a given precursor under different conditions of analysis and among different precursor proteins suggests that the aggregation states depend critically on the experimental conditions, e.g., duration of experiment, temperature, availability of cytosolic factors, or ionic conditions.

Recently, the requirement of a cytoplasmic RNA species for the posttranslational import of mitochondrial precursor protein into mitochondria has been proposed (Firgaira et al., 1984) on the basis of experiments indicating that ribonucleases inhibit the import of the precursor of ornithine transcarbamylase. Apparently, the nucleases act on a ribonucleic acid protein complex which contains the precursor. This complex has an  $M_r \sim 400,000$  as estimated by gel filtration. To date it is neither known if the observed aggregates are homo- or heterooligomers associated with RNA, nor is it clear what role they play in the translocation process. Their formation may protect the precursors against proteolysis (Hay et al., 1984) or in analogy to

the signal recognition particle for secretory proteins (Walter and Blobel, 1982) be involved in the specific translocation of the precursor from its site of synthesis to the mitochondria.

In conclusion, all experimental evidence including the present data indicate that mitochondrial precursor proteins are present in the cytosol as low  $M_r$  forms (homo- or heterooligomers) and as high  $M_r$  forms (possibly as protein-RNA complexes). In the case of mAspAT it is unknown to date whether the low or high  $M_r$  form interacts with the mitochondrial surface and possibly mediates the translocation of the precursor into the organelles. In vitro import assays such as that established in this study (see below) will allow to address this question experimentally.

#### 4.2 In vitro Import into Mitochondria and Processing of pre-mAspAT

In this study the import of pre-mAspAT has been reconstituted in vitro. The precursor was synthesized in a rabbit reticulocyte lysate programmed with free polysomes from chicken liver and incubated with mitochondria isolated from chicken heart. The following lines of experimental evidence confirm that the labeled mAspAT generated during incubation of labeled pre-mAspAT with isolated mitochondria is indeed located in the organelles. (1) The uncoupling agent CCCP dissipating the electrochemical gradient through the inner mitochondrial membrane (Gasser et al., 1982a,b; Schleyer et al., 1982; Kolansky et al., 1982) abolished the appearance of mature mAspAT if present during incubation of pre-mAspAT with intact mitochondria; as expected, CCCP did not inhibit the processing of pre-mAspAT by a mitochondrial extract. (2) Both chelators bathophenanthroline disulfonate and o-phenanthroline (Gasser et al., 1982b) inhibited the processing by

a mitochondrial extract; however, only o-phenanthroline which can diffuse through the inner membrane inhibited the processing by intact mitochondria.

The estimated half-life of pre-mAspAT with respect to import in the in vitro system was  $\sim 30$  min (Fig. 9B), whereas in chicken embryo fibroblasts the half-life of pre-mAspAT was found to be 0.5 min (Jaussi et al., 1982). The in vitro and in vivo conditions were comparable with respect to the amount of mitochondria per unit volume and the concentration of pre-mAspAT. Addition of more mitochondria did not accelerate the process (see Fig. 10A). Similar slow in vitro transport and processing compared to the situation in vivo was observed for pre-ornithine transcarbamylase (Mori et al., 1980;1981), pre-carbamoylphosphate synthetase (Mori et al., 1979;1981) and pre-methylmalonyl-CoA mutase (Fenton et al., 1984). Other precursors have been reported to have somewhat shorter half-lives ranging from 7 to 20 min (Gasser et al., 1982a; Ohta and Schatz, 1984; Schleyer et al., 1982).

The in vitro system is also less efficient than intact cells with respect to the yield of import. Pre-mAspAT which has been accumulated in the cytosol of CCCP-treated chicken fibroblasts, is quantitatively chased into mitochondria on release of the import block (Jaussi et al., 1982). In contrast, only about 50% of the total in vitro synthesized precursor was recovered as mature enzyme on incubation with mitochondria. The bulk of the not imported precursor could be recovered from the assay mixture. It was about equally distributed between the mitochondrial fraction and the supernatant. A minor part of pre-mAspAT had been degraded.

Processing of in vitro synthesized pre-mAspAT to the mature form by a mitochondrial extract was also found to be limited to ~ 50% of the total precursor (Fig. 10B). The coincidence of the yields of import and processing indicates that probably the precursor itself rather than auxiliary factors are limiting. In agreement with this conclusion, addition of more mitochondria, of more reticulocyte lysate, or of a cytosol from chicken heart or fibroblasts all failed to increase the yield of import.

Conceivably, the read-out system might produce in part impaired proteins due to damaged unfinished polypeptide chains in the polysome preparation used. However, this possibility appears unlikely because the same polysome preparation was found to produce a precursor with even lower yield of import and processing in other reticulocyte lysates. Similarly, the finding that precursor from chicken embryo fibroblasts after homogenization of the cells was neither imported into isolated mitochondria nor processed by a mitochondrial extract (not shown) indicated that under cell-free conditions the precursor might be subject to posttranslational alterations preventing its import and processing. Such alterations might include irreversible modifications, irreversible complexation with other cellular constituents, or denaturation. Reportedly, denatured precursors are neither imported nor processed (Böhni et al., 1983).

The possibility to observe the import of pre-mAspAT in a cell-free system allowed to examine whether mAspAT competes with the precursor for its uptake into mitochondria. Excess mAspAT competed with pre-mAspAT neither for the binding to nor the translocation into mitochondria (Fig. 8B). This result emphasizes the importance of the N-terminal extension but does not

prove that the prepiece alone suffices to direct the translocation of pre-mAspAT into the mitochondria. Thus, it cannot be excluded that structural features of the precursor protein other than the N-terminal extension, e.g., the so-called m-residues (see Introduction, 1.2), are involved in the translocation process. Conceivably, the prepiece maintains the precursor protein in a conformation different from that of the mature enzyme, this "nonnative" conformation being required for the binding and/or translocation process. Both mature mitochondrial AspAT and malate dehydrogenase have been reported to be taken up into mitochondria (for a review, see Doonan et al., 1984). In this study, mAspAT was not found to be imported into isolated mitochondria. In the competition experiment the amount of mAspAT recovered from the mitochondrial fraction corresponded to that of endogenous mAspAT present in the control mitochondrial fraction. Moreover, radiolabeled mature mAspAT in a homogenate from chicken embryo fibroblasts into which the enzyme had been released by ultrasonication failed to be imported into isolated fibroblast mitochondria (C.P. Sharma, personal communication).

The N-terminal prepiece of pre-mAspAT is expected to contain basic amino acid residues and to lack acidic residues (see Introduction, 1.1). Excess of polylysine failed to compete with the precursor for its uptake into mitochondria (data not shown). Apparently, not solely positively charged residues are required to address the precursor to the mitochondria.

Douglas et al. (1984) showed that the carboxy-terminal region of the precursor of the  $\beta$ -subunit of  $F_1$ -ATPase is not necessary to target the precursor to mitochondria. A gene coding for the yeast pre- $\beta$ -subunit was fused to the gene encoding E.coli  $\beta$ -galactosidase. Expression of the fusion gene yielded a protein

consisting at its amino terminus of the first 2/3 of the pre- $\beta$ -subunit and at its carboxyl terminus of a large part of the  $\beta$ -galactosidase. The fusion protein was found to cofractionate with mitochondria and protease protection assays (see Results, 3.2) indicated that it had been transported at least through the outer mitochondrial membrane.

Recent data (see Introduction, 1.1) suggest that the N-terminal extension of the precursor of subunit IV of cytochrome c oxidase contains all the information necessary to address even a cytosolic protein, dihydrofolate reductase, into mitochondria. The fusion protein was transported as efficiently as the authentic precursor into the matrix of isolated mitochondria (Hurt et al., 1984a,b). In both cases, half-maximal import and processing was attained after about 30 min. However, the identical rates of in vitro import of the fusion protein and the authentic protein do not necessarily imply that the two proteins are imported at identical rates in vivo. The generally slow rate of in vitro import might mask a difference in the rate of import of the fusion protein and the authentic protein. Further data are needed in order to establish whether for all mitochondrial precursor proteins only the N-terminal extension has a targeting function. The future availability of full-length cDNA clones coding for mAspAT and cAspAT will allow to define which part of the pre-mAspAT molecule directs its binding to the mitochondria and its transmembrane movement.

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